Biofilms on stainless steels exposed to process waters

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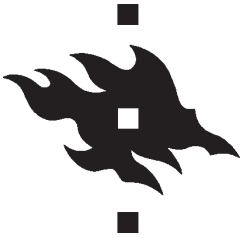

Academic Dissertation in Microbiology

To be presented, with the permission of the Faculty of Agriculture and Forestry of the University of Helsinki, for public criticism in auditorium 2041 at the University of Helsinki Biocenter, Viikinkaari 5, on August 26, at 12 o´clock noon.

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Front cover: Hybridized Baltic Sea biofilm seen by CLSM (Fig 14b) surrounded by paper mill biofilm forming bacteria seen by SEM (Fig 9).

To my family

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Contents

List of original papers

I Mattila K, L Carpén, T Hakkarainen and MS Salkinoja-Salonen. 1997. Biofilm development during ennoblement of stainless steel in Baltic Sea water: a microscopic study. International Biodeterioration and Biodegradation, 40: 1-10.

II Mattila K, L Carpén, L Raaska, H-L Alakomi, T Hakkarainen and MS Salkinoja-Salonen. 2000. Impact of biological factors on the ennoblement of stainless steel in Baltic Sea water. Journal of Industrial Microbiology and Biotechnology, 24: 410-420.

III Mattila K, A Weber and MS Salkinoja-Salonen. 2002. Structure and on-site formation of biofilms in paper machine water flow. Journal of Industrial Microbiology and Biotechnology, 28:268-279.

IV Uutela P, K Mattila, L Carpén, L Raaska, T Hakkarainen and MS Salkinoja-Salonen. 2002. Biogenic thiosulfate and oxalate in paper machine deposits connected to corrosion of stainless steels. International Biodeterioration and Biodegradation, in the press.

The author's contribution

Paper 1:
Katri Mattila carried out all the experimental work except for the open circuit measurements. She interpreted the results, wrote the paper, and prepared all the figures.

Paper 2:
Katri Mattila wrote the paper and is the corresponding author of the paper. She performed all the experimental work except for the open circuit measurements, viable microbial counts, CTC-DAPI stainings, and a part of the acridine orange stainings. She prepared all the figures.

Paper 3:
Katri Mattila wrote the paper and is the corresponding author of the paper. She carried out all the experimental work and prepared all the figures.

Paper 4:
Katri Mattila carried out the epifluorescence microscopy, scanning electron microscopy and elemental analysis. She developed the microbiological part of the experimental set-up, and was responsible for that part of the interpretation.

Abbreviations

AFM	atomic force microscope
ATP	adenosine triphosphate
CBE	Center for Biofilm Engineering, Montana State University
CIP	cleaning in place
CLSM	confocal laser scanning microscopy
CMC	carboxymethyl cellulose
Con A	concanavalin A, in this study labelled with tetramethylrhodamine
CTC	5-cyano-2,3-ditolyl tetrazolium chloride
DAPI	4´,6-diamidino-2-phenylindol
DNA	deoxyribonucleic acid
DSMZ	Deutsche Sammlung von Microorganismen und Zellkulturen GmbH
EDS	energy-dispersive X-ray
EDTA	ethylenediamino tetraacetic acid
EPS	exopolysaccharide
EtBr	ethidium bromide
FESEM	field emission scanning electron microscope
FISH	fluorescent *in situ* hybridization
GFP	green fluorescent protein
HSL	homoserine lactone
log K_{ow}	logarithm of the *n*-octanol: water partition coefficient
LPS	lipopolysaccharide
MIC	microbially influenced corrosion
NA	numerical aperture
OMP	outer membrane proteins
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SEM	scanning electron microscopy
SPM	scanning probe microscope
SRB	sulphate-reducing bacteria
rfu	relative fluorescence unit
TEM	transmission electron microscopy
UNS	unified numbering system

1 Introduction

1.1 Biofilms

During the millions of years when bacteria were the only form of life on Earth, the prevailing aquatic environment was extremely oligotrophic. The niches permissive for life were limited by hostile environmental factors like UV radiation, heat and acidity (Costerton and Lappin-Scott 1995). At that time the purpose of the planktonic, free-living mode of bacterial growth was to enable them to move from one habitat to another until a niche permissive for growth was found. Biofilm formation allowed these first sessile organisms to remain in place, and to trap and utilize the scarce organic compounds. The development of co-operation among bacterial groups permitted the use of more complex or more refractory nutrients. Biofilm formation also changed the environment of the colonized surface and made it more suitable for the bacteria growing there. Biofilm formation has therefore been a means of survival for bacteria, and is the reason why bacterial biofilms are characteristic of the life forms found in extreme environments like stone surfaces in hot springs or the surface of stones in cold, oligotrophic mountain streams. Nowadays this survival strategy is successfully used by bacteria in industrial systems such as drinking water distribution networks, (Szewzyk et al 2000, LeChevallier 1999, Koskinen et al 2000), paper machines (Väisänen et al 1994, 1998, Claus and Müller 1996), dairies (Pirttijärvi et al 1998), breweries (Storgårds 2000) and power plants (Linhardt 1996). In technical systems, biofilm formation can cause problems like the contamination of drinking or process water by micro-organisms, an increase in the flow resistance of pipe lines or a decrease in the thermal transfer capacity in heat exchangers (Flemming 1996). The presence of pathogenic bacteria in biofilms in drinking water distribution systems is a health risk (*Legionella pneumoniae, Klebsiella pneumoniae, Mycobacterium avium*, LeChevallier 1991 and 1999, Szewzyk et al 2000).

Despite the frequent problems caused by biofilms, there are industrial systems where biofilm growth is a beneficial phenomenon. The proper functioning of activated sludge plants, trickling filters or anaerobic digesters is based on functional biofilms. In this thesis the main focus is on undesirable process biofilms.

1.1.1 Heat exchangers and paper machines as biofouling targets

Biofouling is a general term that refers to the undesirable accumulation of biotic deposits on a surface. Microbially induced deterioration occurs in many industrial processes. Damage to metal materials is known to occur in aqueous media, where specific microorganisms can find conditions favourable for growth (temperature, nutrients) (Weber and Knopf 1996). This often happens in heat exchangers and paper machines. Both of these processes use large amounts of natural water, are kept at a constant temperature, and offer a range of substrates for microorganisms to adhere to and grow on.

Heat exchangers as an environment for microbial growth

Industrial coolers are complicated systems of pipes in which excess heat is transferred through metal surfaces to colder, circulating natural water. Cleaning in place (CIP) with

chemicals is the main method used for removing unwanted biofilms. Heat exchangers are usually made of stainless steel to minimize corrosion.

Paper machines as an environment for microbial growth

Paper is made of wood fibre. In addition, paper manufacturing uses water, chemical additives such as fillers, binders, mineral pigments, CMC or starch, and resins or neutral adhesives for sizing. Chipped wood is broken up into wood fibres mechanically (ground wood, mechanical pulp) or chemically (chemical pulp). The pulp is slurried with additives and water to form a "stock" for papermaking (Fig 1). The stock in the head box of a paper machine contains 0.3-0.6 % w/v of fibre and more than 99% water (Biermann 1996). From the head box the stock is spread onto the wire to form a formatted paper web. Water is removed from the web first by gravity, then by suction (consistency 18-23 %) and by pressure (35-55 %), and finally by heat (more than 90 % d.wt, Biermann 1996). Different types of paper machine have been designed to produce different grades of paper. Modern paper machines can be up to 600 m long, and the web up to 14 m wide. The paper web formed on the wire moves at a speed of up to 1500 m min^{-1} (≈ 90 km h^{-1}). The annual production capacity of one paper or board machine in Finland is around 300 000 metric tons.

The presence of water and oxygen in the paper machine, the elevated temperature (30-55ºC), and suitable pH conditions (4-10), permit the growth of many kinds of microorganism (Väisänen et al 1994 and 1998, Lindberg et al 2001a, Kolari et al 2001, Busse et al 2002). Bacteria, moulds and yeasts may deteriorate the raw materials used in paper making (Väisänen et al 1998, Pirttijärvi 2000 and 2001, Salzburger 1996), form biofilms which clog screens, wires and felts, or generate metabolic products that are corrosive or otherwise deteriorate the machinery.

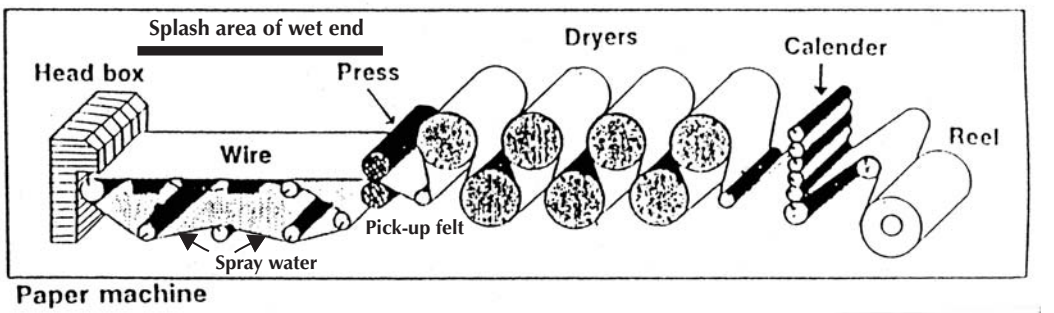


Figure 1. Basics of a paper machine. Paper making stock is pumped into a head box, from where it is spread onto a moving wire (=finely woven plastic web). Water drains through the wire by gravity and suction. The fibres remain on the wire to form a paper web. Fibres tend to align themselves in the direction of movement of the wire (=machine direction). The paper web at this stage contains 80 to 90 % of water. The wire returning to the head box is washed with a pressurized spray water. The paper web is pressed between dryer felts and up to press roll nips. Between the two pressuring rolls water is pressed from the web to reach a dry matter content of ca. 40 % w/w. Pressing improves inter-fibre bonding by bringing the fibres closer together. The paper web then passes between several steam-heated drying cylinders until the dry weight of ≥ 90 % is reached. This drying is the most energy consuming part of papermaking. After drying the paper is wound onto spools to form machine rolls (modified from Papermaking, FINNPAP lecture material, Helsinki, Finland, 1995).

The environment inside the paper machines and heat exchangers may promote the growth of certain microbial species at specific locations, even though the overall conditions in the installations do not favour such microorganisms (Weber and Knopf 1996). Biofilm formation is one of the most common causes for a local change in the environment inside industrial processes using water.

1.1.2 Biofilm: growth on surfaces

Non-living surfaces in an aqueous environment rapidly accumulate organic molecules and inorganic ions to form a layer called the ”conditioning film” (Fig 2). Therefore, in all but the most oligotrophic ecosystems, planktonic bacteria actually adhere to the surface of a surface film that may have different chemical properties than the non-living surface (Costerton and Lappin-Scott 1995, Marshall 1997). The initial attachment of bacteria to a surface frequently involves a portion of the cell, a flagellum or EPS, while the bacterial cell continues to revolve (Marshall 1988). During so-called reversible attachment, the bacteria use its motility to sustain contact with the surface while searching for a suitable location there (Korber et al 1995). Such a search is also called chemosensoring if the bacteria prefer specific substrates present on the surface or produced by other

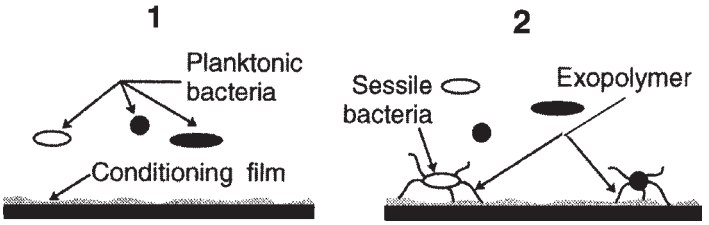


Figure 2. A) The initiation of biofilm growth. At stage 1 a conditioning film accumulates on a submerged surface. Later, at stage 2, planktonic bacteria from the bulk water colonize the surface and begin a sessile existence by excreting exopolymers anchoring the cells to the surface (modified from Geesey 1993).

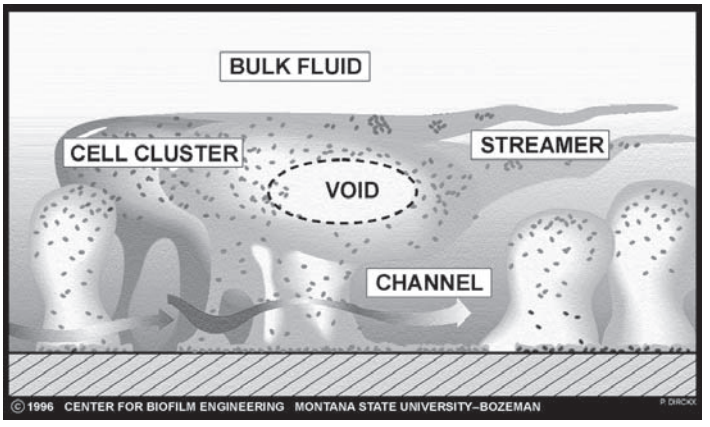


Figure 2. B) Conceptual model of a mature bacterial biofilm drawn on the basis of CLSM examinations of a large number of mono- and mixed-species biofilms. The discrete microcolonies of microorganisms are surrounded by a network of interstitial voids filled with water. The arrows indicate convective flow in the water channels (Costerton et al 1994, Courtesy of Center for Biofilm Engineering, Montana State University, Bozeman).

bacteria (Nielsen et al 2000). Methods of surface translocation like the flagellar motility of bacteria (Davey and O'Toole 2000, Martínez et al 1999, McBride 2001), are called positioning mechanisms.

Flagellum-mediated motility is required in *Escherichia coli* for both approaching and moving across the surface. Type I pili and outer membrane proteins are required for a stable organism-surface interaction in *E. coli* (Fig 3). *Pseudomonas aeruginosa* uses its flagellum only for bringing the cell into the proximity of a surface (Davey and O'Toole 2000). The initially reversible attachment may transform into an irreversible one, or to detachment of the bacterial cell. Reversible attachment is considered to be predominant in nature (Korber et al 1995).

The initial attachment of a single bacterium is based on interaction between the cell and the substratum. The subsequent growth and maturation of the biofilm depends on cell-to-cell interactions called coaggregation. Coaggregation can be defined as "the recognition and adhesion between genetically distinct bacteria" (Whittaker et al 1996). The formation of a biofilm cluster, coaggregation, may be regulated by means of signalling systems between cells sharing the same location, irrespective of whether they are related or not (Xie et al 2000).

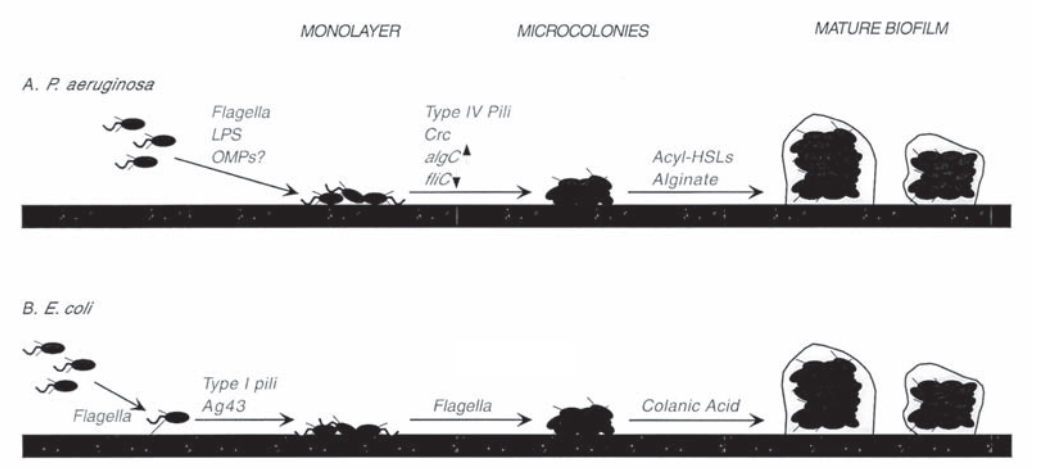


Figure 3. Development of biofilm by gram-negative bacteria. This figure outlines the current model for the early stages in biofilm formation in P. aeruginosa and E. coli.
A) In P. aeruginosa, flagella are required to bring the bacterium into the proximity of a surface. LPS mediates early interactions, with an additional possible role of outer membrane proteins (OMPs). Once the bacteria are in a monolayer on the surface, type IV pilus-mediated twitching motility is required for the cells to aggregate into microcolonies. The documented changes in gene expression at this early stage include the upregulation of alginate biosynthesis genes and downregulation of flagellar synthesis. Production of cell-to-cell signalling molecules (acyl-HSLs) is required for the formation of a mature biofilm. Alginate may also play a structural role in this process.
B) In E.coli, flagellum mediated swimming is required for both approaching and moving across the surface. Organisms-surface interactions require type I pili and the outer membrane protein Ag43. Finally, EPS known as colanic acid is required for development of the normal E. coli biofilm architecture (modified from Davey et al 2000).

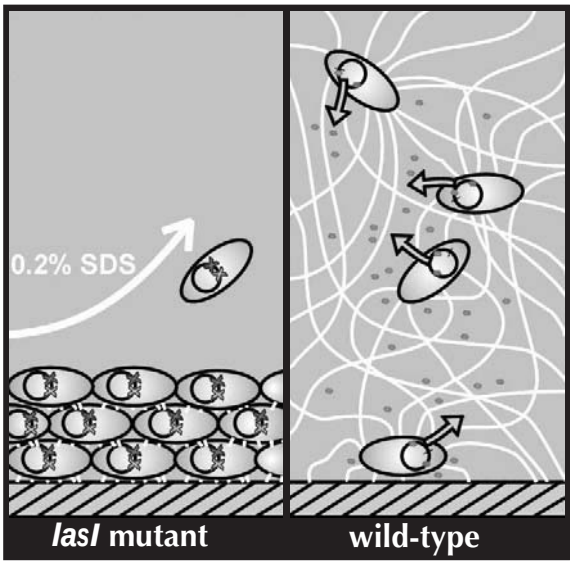


Figure 4. Characteristics of P. aeruginosa wild type and quorum-sensing mutant biofilms. lasI mutant forms an evenly distributed biofilm, which was detached and dispersed in 5 min following treatment with sodium dodecyl sulfate (SDS, 0.2%) (Courtesy of Center for Biofilm Engineering, Montana State University, Bozeman).

The role of quorum sensing in biofilm formation

Quorum sensing means cell-to-cell signalling, and is an important phenomenon of microbial communities. Recent results (Davies et al 1998, Whitehead et al 2001) show that it regulates biofilm formation. Two quorum sensing systems have been described for *Pseudomonas aeruginosa (*Davies et al 1998, Wimpenny et al 2000): *lasR-lasI,* which controls virulence but also regulates the expression of a second system, and *rhlR-rhlI,* which is involved in the formation of secondary metabolites. These systems control the production of the signal molecules: butyryl homoserine lactone by *rhlI,* and 3-oxododecanoyl-homoserine lactone by *lasI.*

The wild type and a *lasI* mutant both attached themselves to a surface, whereas the *lasI* mutant retained its planktonic behaviour by forming a thin, evenly distributed biofilm that was dispersed in 5 min by detergent treatment (SDS, 0.2%) (Davies et al 1998, Fig 4). Wild type cells of *P. aeruginosa* formed characteristic microcolonies composed of groups of cells separated by water channels. Similar microcolonies were formed by the *lasI* mutant, but only after addition of an autoinducer (3-oxododecanoyl-homoserine lactone).

Quorum sensing requires a sufficient density of bacteria. For this reason, neither of the *P. aeruginosa* signal molecules would be expected to participate in the initial stages of biofilm formation, i.e. attachment or proliferation. Instead, these quorum sensing signals may be involved in biofilm differentiation. The results suggest that the initial stages of biofilm formation in the *lasI* mutant, proceed in a similar fashion to that of the wild type, but that differentiation from attached planktonic cells into biofilm bacteria was blocked.

This field has been intensively investigated ever since the first regulatory signalling system was found (Shapiro 1998, Kolenbrander 2000, Miller and Bassler 2001, Whitehead et al 2001, Schauder and Bassler 2001). Quorum sensing systems have been found to play an important role in mature biofilm communities. Signalling systems regulate the morphological transformation of bacteria when they become a permanent part of the biofilm: flagellin synthesis is decreased, and the production of exopolysaccharide is

increased (Watnick and Kolter, 2000). Polysaccharide production is favoured since it reinforces the biofilm structure (Danese et al 2000). Cells located in the centre of a biofilm cluster do not divide at all, or divide only slowly. However, they remain viable and culturable once freed from the plastic encasement of the biofilm cluster (Geesey 2001).

Bacterial products diffusing from one cell to another cell may be responsible for intercellular communication. The reason why metabolic communication, like quorum sensing, is not efficient in the case of planktonic bacteria, is that the diffusing molecules will be diluted in the aqueous phase and only a small amount may reach the neighbouring bacteria (Davey and O´Toole, 2000).

Factors influencing the attachment of bacteria onto solid substrata

The major factors regulating attachment or detachment of bacteria onto or from surfaces are nutrient availability, the electrochemical properties of the surface, and liquid flow. When nutrients are non-limiting in the liquid phase there is no need for the bacteria to attach themselves. Stress situations like a depletion of nutrients makes sessile growth more favourable in flowing liquids (O'Toole et al 2000). Adding biocides at sublethal concentrations into process waters may be a stress factor that initiates biofilm formation (M. Kolari, unpublished data), even though the desired effect was the opposite. Adherence to a substratum is favoured in cases where the substratum could be used as a source for nutrients (Watnick et al 1999). "Over" growth of a biofilm cluster may cause detachment. Free-living bacteria will search for a favourable environment in which to reattach themselves.

The attachment to non-living surfaces depends on the physicochemical properties of both the bacteria and the substratum (Dalton et al 1994). Non-living surfaces can be divided into two main classes based on their surface energy: 1) High-energy surfaces, which are usually also hydrophilic and frequently negatively charged. These include inorganic materials such as glass, non-noble metals or minerals. 2) Low-energy surfaces are often hydrophobic and have a low negative or positive electrostatic charge. These include synthetic polymers, noble metals and high quality stainless steels. Because of the high surface activity, high-energy substrata readily adsorb dissolved solutes or atmospheric contaminants and are therefore rarely clean (Fletcher 1990). Contaminants that form a conditioning layer change the properties of the naked substratum surface and therefore also the surface charge. Bacteria may change their surface composition in response to the environment. The degree of surface hydrophobicity of the microbial cell has been used to predict attachment phenomena. However, its relative importance is likely to be low because 1) no clear trend has been found between cell attachment and the measured surface hydrophobicity, and 2) cell surface hydrophobicity is not necessarily constant for a given organism (Scheuerman et al 1998).

The topography of the substratum and the liquid flow influence the attachment of bacteria. It is assumed that the smoother the surface topography and the higher the liquid flow, the more difficult it is for microbes to become attached. Scheuerman et al (1998) reported that the presence of grooves in a surface, running perpendicular to the direction of the flow, resulted in preferential attachment of bacteria to the downstream edges of the grooves, and to a lesser extent to the upstream edges. Non-motile bacteria were not found in the bottom of the grooves. An increase in flow rate (from 28 mm s^{-1} to 83 mm s^{-1}) promoted attachment and decreased the importance

of the positioning of the cells relative to the grooves. The flow rate recommended by Geesey (1993) for the prevention of bacterial attachment and subsequent risk of corrosion is > 1.5 m s^{-1}. According to Stoodley et al (1999), a mixed-species biofilm grown in a laminar flow consisted of roughly circular-shaped microcolonies separated by water channels. In contrast, biofilm microcolonies grown in a turbulent flow were elongated in the downstream direction, forming filamentous "streamers". These extensive, ripple-like structures allowed the biofilm to migrate downstream.

Mature biofilm and detachment

Biofilms that have reached a stable size (thickness), i.e. where growth is compensated by detachment, are called mature. Mature biofilms can have a complex architecture. Early studies on biofilms by electron microscopy required dehydration of the specimens, leading to a deceivingly simplistic view of biofilms as cells piled on top of one another (Costerton et al 1995). Recent advances in confocal scanning laser microscopy have allowed visual inspection of fully hydrated biofilms. Visual observations of the biofilm interior have been connected to chemical data with microelectrode studies (Rodrigues et al 1992, DeBeer et al 1997, Costerton et al 1994 and Santergoeds et al 1999). The use of a microelectrode with a small diameter tip (down to 10 mm) enabled separation of chemical data obtained either from the biofilm clusters or the void space seen by CLSM. This has radically changed our views of biofilm architecture (Lawrence et al 1991, O'Toole et al 2000). Mature biofilms are highly hydrated open structures containing a high proportion of exopolymers and large void spaces between the microcolonies (Fig 2b) (Lawrence et al 1991). The void space will remain uncolonized if there is a low substrate concentration or/and a high signal molecule concentration. Similarly, a high concentration of leaking metabolites will direct the free-swimming bacteria towards the microcolonies (Tolker-Nielsen et al 2000b). Stewart (1993) divided biofilm detachment processes into four categories: abrasion, erosion, sloughing and predator grazing. Abrasion and erosion both refer to the removal of small groups of cells from the surface of a biofilm. Sloughing, in contrast, refers to the detachment of large portions of the biofilm, which may reach or be even wider than the thickness of the biofilm itself (Morgenroth and Wilderer, 2000). It is also likely that detached, large biofilm clusters will retain their protective properties against antimicrobials while in the liquid phase (Stoodley et al 2001).

1.1.3 Cultivation of biofilms

The potential of bacteria to form biofilms can be measured in the laboratory using microtiter plates (Danese et al 2000, Fletcher 1990, Kolari et al 2001). The growth medium and the bacterial inoculum are dispensed in the wells of the plate, and incubated at a chosen shaking rate and temperature for a specific period of time. The wells are then emptied, washed and the biofilm that has accumulated on the walls of the wells is stained (e.g. crystal violet). The colour intensity of the attached cells can be measured by a plate reader. This method is simple, and allows a large number of analyses to be carried out simultaneously. However, there are also drawbacks to this technique. Commercially available substrata (microtiter plates) are limited to a number of different types of polystyrene. Furthermore, insufficient shaking during incubation or subsequent washings may lead to sedimentation of the bacterial cells and false positive results.

When the development of biofilms needs to be followed on-line, flow cells (flow slides) connected to a CLSM can be used (Kuehn et al 1998, Wolfaardt et al 1994). In this technique, the bacteria form a

biofilm under continuous flow conditions on the interior surfaces of a thin flow chamber, placed directly under the microscope. Formation of the biofilm is visualised by the attenuation of transmitted light or fluorescence when bacteria carrying the green fluorescent protein (GFP) are used (Martínez et al 1999, Kuehn et al 1998), or by staining the bacteria with non-destructive, non-toxic fluorescent stains.
Robbin's devices are flow cells designed for use in industrial applications (Ladd and Costerton 1990, Blanco et al 1996, Elvers et al 1998). In this technique, process water is allowed to flow through the device and a biofilm is formed on exchangeable surfaces, so-called test coupons. The flow cells have to be sufficiently robust to operate in industrial systems. Surfaces of different composition or quality and a range of flow rates can be used with Robbin's devices. The test coupons can be removed from the device and the biofilms formed on the coupons can be chemically or biologically analysed.

A range of other types of equipment have also been developed for biofilm studies: e.g. a rotating disc reactor (CBE, Montana referred by Murga et al 2001) and Calgary biofilm device (Ceri et al 1999), which is modified 96 well microtiter plate system.

1.1.4 Methods for biofilm characterization

The confocal laser scanning microscope

The brilliant idea of combining a powerful laser beam as a point light source and the use of pinholes to cut off the non-focal light has completely changed the fluorescence microscopy of biological specimens. In a confocal laser scanning microscope (CLSM), confocal pinholes in the light source (laser) and in the detector eliminate light from above and below the plane of focus. The laser then scans in x-y directions through the specimen at different planes (z-direction) of focus. A 3-D image is built from the information gathered by the detector (Fig 5). As a result, each single x-y image is an optodigital thin section. The thickness of each section depends on the lenses used and on the size of the confocal pinhole, and approaches the theoretical resolution of the light microscope (≈ 0.2 μm) (Pawley 1995b, Caldwell et al 1992). For example, when lenses with an NA of ≤ 0.2 are used, the thickness of the optical thin section is approximately 10 μm. However, this value rapidly drops to less than 1 μm when lenses with an NA of > 0.6 are used. The use of laser beams also means that the sample can be scanned in the sagittal (zx) plane down to the depth of the laser light penetration, which is dependent on the opacity and optical homogeneity of the specimen (Centonze and Pawley, 1995). Lawrence et al (1997) reported effective sectioning through 1 mm biofilm material with 40 X water immersible lenses. If a cover glass is used, the thickness of the sectioned biofilm is smaller because the thickness of the cover glass is included in the sectioning distance. CLSM analysis requires no reagents other than aqueous solutions of fluorescent dyes, and can be performed on fully hydrated, living biomass. This is the basis for constructing 3-D images of living biofilms.

The use of digital image collection allows the combination of fluorescence images from separate scans made at different wavelengths of laser light. For instance, fluorescent phylogenetic probes can be used as tools for documenting the spatial distribution of chosen species or genera of bacteria, archaea or eukaryotic micro-organisms in a microbial community (Stewart et al 1997, Tolker-Nielsen and Molin, 2000a). It also allows recognition of the EPS formed by the biofilm cells, as well as visualization of the co-operation between several bacterial species within the biofilm

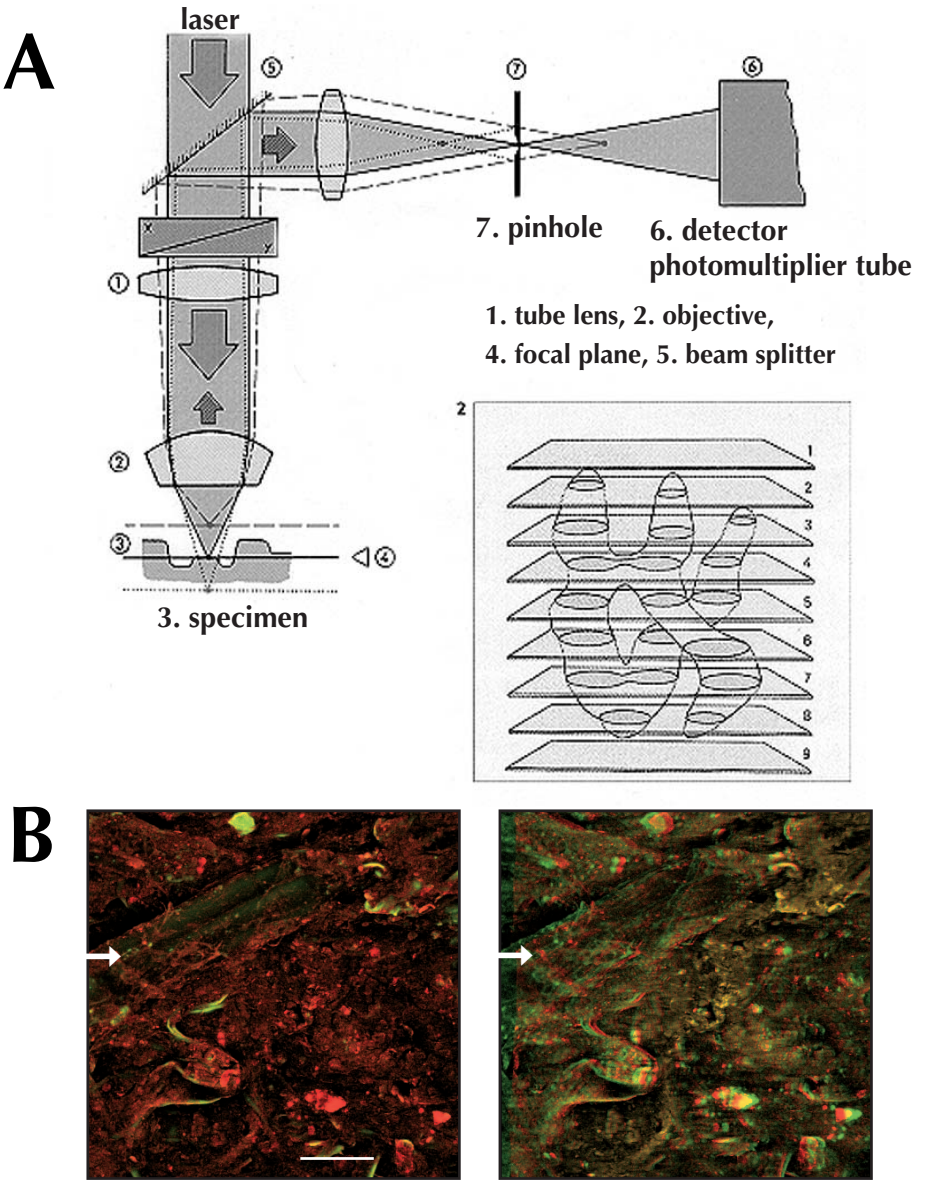


Figure 5. Functioning of the confocal laser scanning microscope.
A) A laser light beam is focused to a single point in the specimen. The pinhole (7) for excited light determines the thickness of the focused layer (4); the smaller the pinhole, the thinner the focus layer. During the scanning of the layer in focus the emission information is gathered by the detector (6, photomultiplier tube) and transformed to an image on the computer screen. After collection of information from several layers of focus, the data can be combined and 3 dimensional pictures of specimen can be generated (modified from Zeiss Information with Jena Review, No. 5/95).
B) CLSM image of the surface of paper stained with acridine orange. The panel on right side is a combination of 40 optical sections (0.5 μm). Bacteria are the small green dots on surface of wood fibre in the paper. The left panel is a 3-D stereo reconstruction of the same pile of optical sections. The differences in depth are more clearly visible. Measure bar is 10 μm.

consortium (Manz et al 1999). The green fluorescent protein (GFP) has been used for localizing chimeric proteins (Phillips 2001). Rice et al (2000) studied "primary" biofilm formation and the progeny of the first generation of sessile cells, i.e. the "secondary" biofilm cells, by *Pseudomonas aeruginosa* PAO1 producing green fluorescent protein. CLSM can also be used to detect heavy metals. Wuertz et al (2000) used the complexing agent Newport Green, which fluoresces upon binding to Ni, Zn or Co, to detect these metals in 3-D bacterial biofilms. Table 1 compiles the applications in which CLSM is frequently used. For more detailed information on nearly all aspects of confocal microscopy, the Handbook of Biological Confocal Laser Microscopy (Pawley 1995a) is an excellent source.

Study of biofilm microbial communities by in situ hybridization

Although in itself a powerful tool, the development of specific fluorescent nucleic

Table 1 Applications of confocal laser scanning microscopy for biofilm analysis

Target	Tool	Method	Reference
Presence and quality of bacteria			
Nucleic acid staining	Distinguish bacteria from other attached substances	Syto 16, EtBr	Kolari et al 1998
Fluorescence in situ hybridization FISH	Detect bacteria belonging to a specific phylogenetic group	Fluorescently labelled oligo nucleotides	Amann et al 1992, Amann et al 1995, Zarda et al 1997, Hristova et al 2000, Amann and Schleifer 2001
Immuno-fluorescence	Detecting specific antigens on microbial cell surface	Fluorescently labelled mono- or polyconal antibodies	Bohlool and Schmidt 1980
Functional staining	Detection of vital functions of bacteria	CTC-DAPI, Live-Dead	Bredholt et al 1999, Maukonen et al 2000
Properties of biofilm			
Presence of exopoly-saccharide (EPS)	Analysis of EPS components	Fluorescently tagged lectins	This thesis
Biofilm architecture	Detection of pores and channels in biofilm	Fluorescent latex beads, Dextrans	Kolari et al 1998, Stoodley et al 1994, Lawrence et al 1994

acid probes has added a high degree of specificity to CLSM. Such probes allow the detection and identification of individual organisms at levels from phylum right down to strain (Wimpenny 2000). The only disadvantage is that the fully hydrated biofilms cannot be hybridized. The cell membranes of the bacteria, forming the biofilms, have to be broken in order to allow entry of the probes. The term *in situ* hybridization implies that whole cells are hybridized and that the cells are viewed in their natural microhabitat (Amann 1995). Different approaches using nucleic acid probes are presented in Fig 6a. An earlier approach based on immunofluorescence proved that fluorescent signals allow high resolution and fast detection by epifluorescence microscopy. The fluorescently monolabeled, rRNA-targeted oligonucleotide probes were then shown to allow the detection of individual microbial cells (DeLong et al 1989). This made whole-cell hybridization with rRNA probes a tool suitable for determinative, phylogenetic and environmental studies in microbiology (Amann et al 1990, Amann et al 1995, Amann and Schleifer 2001, Rabus et al 1996, Zarda et al 1997, Manz et al 1998, Kalmbach et al 2000, Hristova et al 2000).

The method is based on the use of rRNA-targetted oligonucleotide probes. The

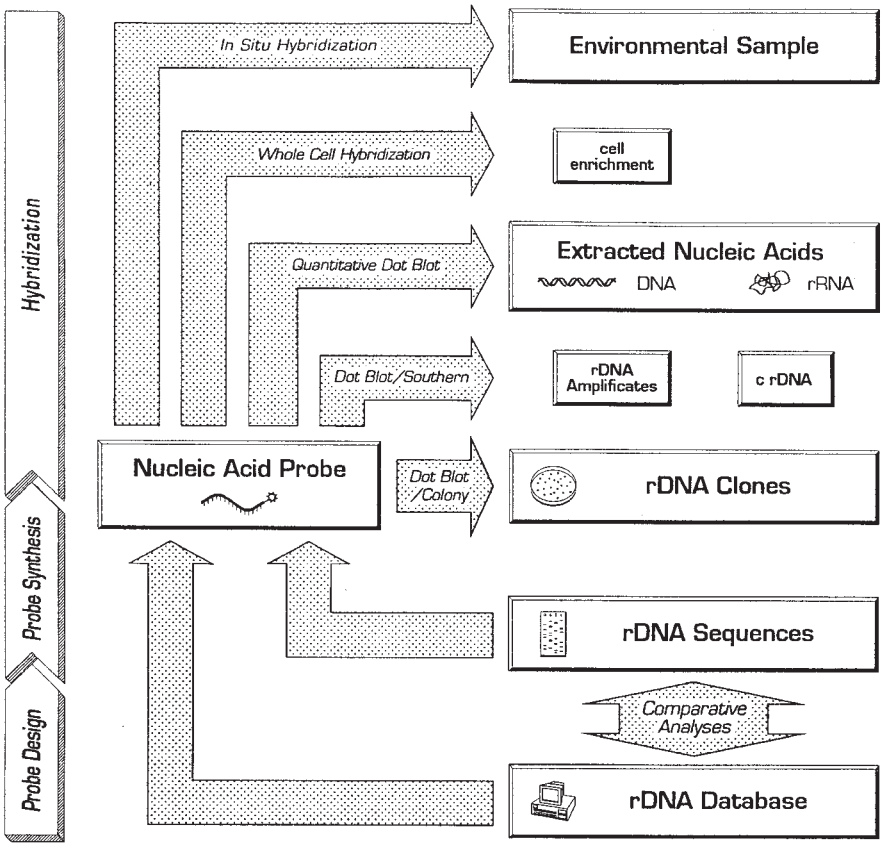


Figure 6. A) Flow chart showing the different options of using rRNA-targetted nucleic acid probes to detect the presence of different taxa in an environmental sample by in situ hybridization techniques (Amann et al. 1995).

probes are usually 17 to 19 bases long. As rRNA sequences are a patchwork of evolutionary conserved regions, signature sites can be identified, and probes designed, for any taxon between the level of the domains *Archaea, Bacteria, Eucarya* and single species. Computer programs are available to generate probes based on the sequence data obtainable from public databases (Amann and Schleifer 2001). Fuchs et al (1998) showed that the target site placement in the secondary structure of rRNA strongly influences the intensity of the fluorescence emitted by the probe (Fig 6b). Several areas are so shaded that access of the hybridization probe to the target site during hybridization is hindered.

Scanning electron microscopy as a tool to study biofilms

Scanning electron microscopy (SEM) has for long been used for determining micrometer-scale details from dehydrated

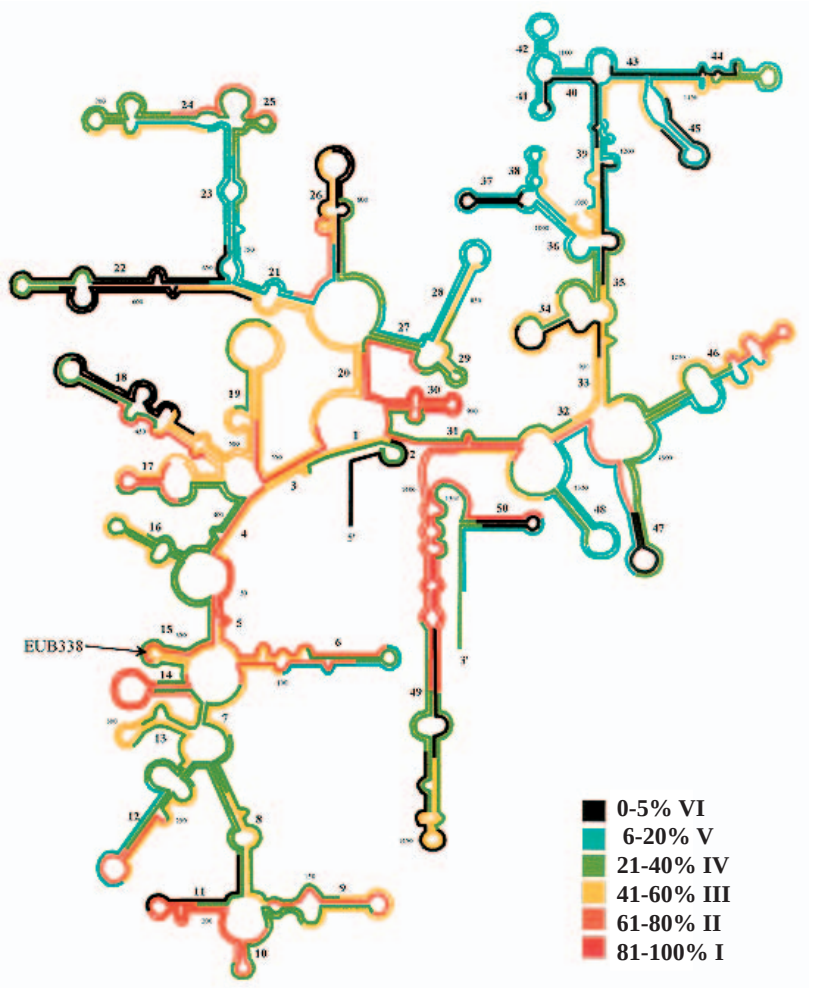


Figure 6 B) Distribution of relative fluorescence intensities of oligonucleotide probes, standardized to that of the brightest probe, Eco1482, on a 16S rRNA secondary structure model. Different colours indicate different classes, decreasing in brightness from I (red) to VI (black) (Fuchs et al 1998).

biological specimens. Chemical fixation and dehydration to allow imaging under a high vacuum, are needed to preserve the cellular structures. The final drying is in air or by critical point drying (Lounatmaa and Rantala 1991). As biological specimens consist of light elements that are inefficient in scattering the electron beam, shadowing by vaporised platinum or gold is usually carried out in order to generate an electron-dense surface (Table 2). The steps performed prior to the microscopic inspection inevitably modify the fine structure of the biological specimens by causing shrinkage or by forming artefacts.

Energy dispersive X-ray analysis (EDS) for analysing elemental composition

Scanning electron microscopes are frequently equipped with an energy dispersive x-ray analyser. This equipment permits elemental analysis with a high horizontal resolution of the inspected

Table 2 Electron microscopic and atomic force microscopic methods for biofilm analysis

Morphology of biofilms			
tool	**method**	**application**	**reference**
Scanning electron microscopy SEM	Sample surface coated with electron dense material is scanned with an electron beam in vacuum	Distribution and morphology of bacteria	Väisänen et al 1998
Transmission electron microscopy TEM	Sample embedded in polymer resin is sliced to thin (0.1 to 2 mm) sections. Penetration of electron beam through the thin section is visualized	Distribution of bacteria and other substances in side of biofilm	Väisänen et al 1994, Kostyal 1998
Scanning probe microscopy SPM, Atomic force microscopy AFM	Mechanical sensor tip scans the surface topography of cells and measures the van der Waals forces towards or away from the tip	Nano resolution picture of living material	Dufrêne 2001, Telegdi et al. 1998, Razatos et al 1998, Kolari et al 2002
Field emission scanning electron microscopy FESEM	Field emission instead of normal wolfram emission allowing a narrower beam of electrons to be used.	High magnification (10000 x) imaging of bacteria distribution and morphology	Kolari et al 2001
Distribution of chemical elements in biofilms			
tool	**method**	**application**	**reference**
Energy dispersive X-ray analysis EDS combined with SEM or TEM	Elemental characteristic energy changes are recorded as x-rays emitted by atoms in the specimen after electron bombing.	Elemental composition of specimen	Gharieb et al 1998, Sayer et al 1997, Otero et al 1997, Nurmiaho-Lassila et al 1990

specimens. Changes in the electron energy levels (emitted x-rays) of the elements are transformed to elemental distribution information (Lounatmaa and Rantala 1991). The vertical depth of the analysis is dependent on the voltage used. As biological specimens are usually easily permeable to electrons, signals from the substratum will also be detected. Light elements like carbon, nitrogen, phosphorus and oxygen are important in biological studies, and they can be detected with high quality analysers (Sivonen 1991).

2 Biofilm-driven processes

2.1 Biofouling

Biofouling is a general term referring to the undesirable accumulation of biotic deposits on a surface. The deposit may contain micro- and macro-organisms (Characklis 1991). Here we focus on microbial fouling, i.e. biofilms where microorganisms are the main biota in an organic film. The organisms may be embedded in a polymer matrix of their own making, or one from an external source. Biofilms may also contain non-microbial materials, such as process "dirt". All material adhering to a steel surface hampers cleaning and may serve as a substrate for microorganisms. Protozoa grazing on microorganisms may also occur in biofilms (Pedersen 1982).

How could biofouling be prevented in industrial systems? Mechanical cleaning is the oldest way to keep systems working. Usually the process needs to be shut down for mechanical cleaning and the down time is expensive. More modern cleaning methods are CIP (cleaning in place) and the use of biocides (Paulus 1993, Rossmoore 1995) to kill microbes present in process water (Wirtanen et al 1995) while the system is operating. However, the problem with this approach is that biocides are usually very effective against planktonic bacteria but less effective against biofilm bacteria (Brown and Gilbert 1993). Biocide efficiency can be improved by using dispersants and other additives targeted against biofilm bacteria or their polymers. Physical means for preventing attachment are high flow rates (> 1.5 m s^{-1}, Geesey 1993) and the use of "fouling release surfaces", which rely on a low surface energy to inhibit strong attachment (Ista et al 1999). Temperatures above > 60°C (Korkhaus et al 1996) have also been used to attenuate microbial growth and biofouling.

Measurement of the biomass attached to surfaces

The degree of biofouling can be expressed as the quantity of biomass covering a given area of surface (Table 3). The metabolic activity of the adhered microorganisms can also be used to measure biofouling. The most straightforward technique is to determine the ATP content of the biomass (Fletcher 1990). ATP production is subject to homeostatic regulation and an expensive process for the cells. Therefore the content of ATP in living microbial cells is relatively constant (ca. 0.02-0.1 % of dry weight, Salkinoja-Salonen 2002). ATP can be extracted from the cells and quantified using the luciferase-luciferin enzyme system of the firefly. The bioluminescence is then measured by a luminometer. The luminometer recording is proportional to the concentration of ATP in the extract, and ATP can be determined down to quantities of 0.01 to 0.1 fmol (Gregg 1991). As the biofilm extract is likely to include inhibitors of luminescence, an internal standard must be used. ATP measurement has been used to replace viable cell counts and for the assessment of surface hygiene (Bretholt et al 1999, Maukonen et al 2000, Stone et al 1999).

Table 3 Methods for estimating biomass quantities on non-living surfaces

Assay		**method**	**reference**
Measure of biomass by content of ATP	direct	Extraction of ATP by boiling in Tris EDTA and measuring the contents of ATP by light production using luciferase enzyme	This thesis
	by swapping	Swapping the surface with alginate swap and dissolving the ATP from the swap to be measured as light produced by luciferase enzyme	Davidson et al 1999
Gravimetric analysis	wet or dry weight	Substratum with biofilm is weighed	
Plate count	swapping and plating	The surface is swapped with alginate swap and bacteria are diluted to maximal recovery liquid and plated on agar. Colony forming units are counted	Carpén et al 1999
Fluorescence	microscopy	Bacteria are stained on surface and counted as number of cells or coverage of biofilm. Acridine orange (total biomass), CTC+DAPI (activity measurement)	Schaule and Flemming 1996, Fletcher 1990 Stewart et al 1994, Yu et al 1994
	fluorometric measurement	Bacteria are stained on surface and counted by analyzer in units of emitted fluorescence	This thesis

2.2 Biocorrosion

Microorganisms growing on a surface perform a variety of metabolic reactions, the products of which may promote deterioration of the underlying substratum (Geesey 1991, Dowling and Guezennec 1997) (Table 4). The main focus of the present thesis is laid on reactions where the substratum is of metal or of metal alloy. How does biocorrosion differ from "mere corrosion"? Flemming (1996) suggested that microorganisms do not introduce a "new" corrosion mechanism, but they can speed up the chemical and electrochemical corrosion kinetics.

Besides the fact that microorganisms and their metabolic products directly trigger and accelerate corrosion processes, their presence in technical systems causes the formation of layers of variable thickness, generating cells with different oxygen contents (Fig. 7a). Such cells drive the dissolution of metals. It does not matter whether the differential aeration cells were generated by microorganisms or by non-living deposits (Weber and Knopf 1996). In addition, bacterial metabolism may produce aggressive substances, oxidants, reductants, acids and complexing agents (Fig. 7b). This may lead to local patches of low pH and/or redox potential underneath the biofilm cluster and promote corrosion by solubilizing the metals by complex formation (Dowling and Guezennec 1997).

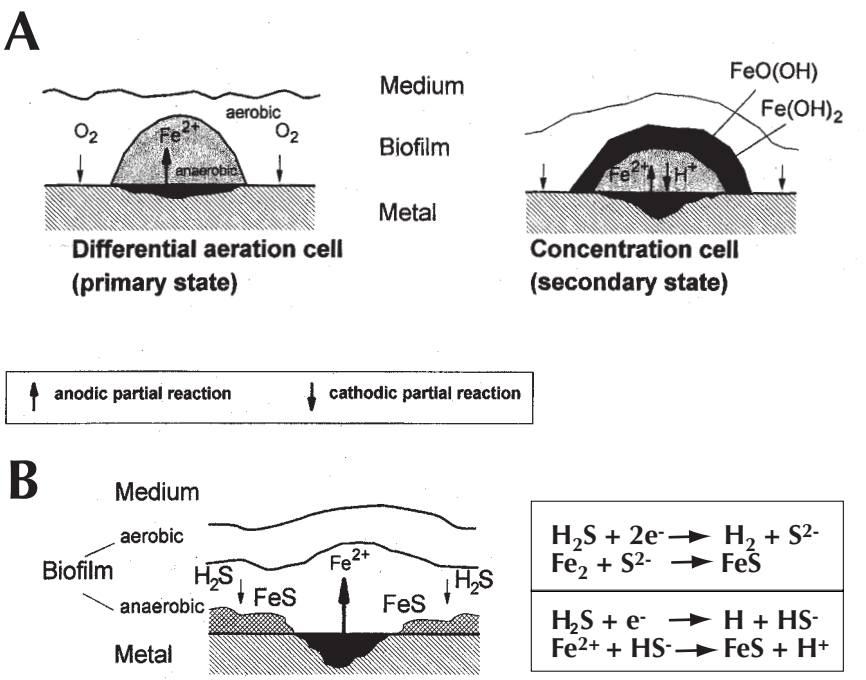


Figure 7. A) Localization of corrosive attack by concentration cells. B) Corrosion process in the presence of sulfides. The arrows up and down show the anodic and cathodic partial reactions (modified from Heitz 1996).

Methods for the detection of (bio)corrosion

The simplest method for determining whether surfaces in a process are corroded or not is to take a sample, clean its surface and inspect it with a light and /or scanning electron microscope. Removal of oxides and other deposits before inspection is necessary because the pits are frequently situated below the deposits (Tatnall 1991). The pits are commonly in the shape of a pocket or crevice. The entrance on a steel surface may be small, but the underlying cavity may become large. Exchangeable corrosion probes can also be used in process water systems to monitor for corrosion (Tatnall 1991). When stainless steel is immersed in natural water the open circuit potential of the steel may increase during biofilm formation. This potential shift towards the noble direction is called ennoblement, and indicates that corrosion may subsequently follow (Anonymous 1995, Scotto and Lai 1998, Carpén et al 1995, 1997a, 1997b, Korkhaus et al 1996, Hakkarainen et al 1996a, 1996b).

Table 4 Bacterial taxa connected with corrosion

Genus or species	reaction	reference
Sulphate and thiosulphate reducing bacteria		
Desulfovibrio desulfuricans	$4H_2+SO_4^{2-}+2H^+ \rightarrow H_2S+4H_2O$	Kuenen 1999
Desulfovibrio	Corrosion of stainless steel pipes	Otero et al 1997
Sulphide and sulphur oxidizing bacteria		
Thiobacillus thiooxidans	$2S^0+3O_2+2H_2O \rightarrow 2SO_4^{2-}+4H^+$	Kuenen 1999
Thiocapsa	Corrosion of stainless steel pipes	Otero et al 1997
Iron oxidizing or reducing bacteria		
Thiobacillus ferrooxidans	$4Fe+O_2+4H^+ \rightarrow 4Fe^{3+}+2H_2O$	Kuenen 1999
Thiobacillus intermedius	Corrosion of iron surface	Telegdi et al 1998
Sphaerotilus sp.	Corrosion of carbon steel	Starosvetsky et al 2001
Gallionella	Corrosion of stainless steel pipes	Tatnall 1991
Siderocapsa	Corrosion of stainless steel pipes	Geesey 1991
Manganese oxidizing bacteria		
Leptothrix sp.	$2Mn^{2+}+O_2+2H_2O \rightarrow MnO_2+4H^+$	Kuenen 1999
Hydrogen forming bacteria		
Clostridium	Hydrogen embrittlement	Geesey 1991

2 Aims

The specific aims of this study were to:

I Determine the sequence of biological events that occur when a biofilm is formed on stainless steel during its ennoblement in Baltic Sea water.

II Identify environmental factors that influence the biology-driven ennoblement of stainless steel in Baltic Sea water.

III Develop a high fidelity laboratory simulator for the development of microbial communities in the splash area of a paper machine. This was done in order to gather information on the salient features of the biological events that are involved in deposit formation related to the corrosion of printing paper machine steels.

IV Analyse biofilms and their formation in the paper machine *in situ*, using the wet end area as an example.

3 Materials and methods

The methods described in detail elsewhere are compiled in Table 5. The fluorescent stains used in this study are listed in Table 6.

3.1 Stainless steel types

Stainless steel was used as the substratum for biofilm growth in natural (Baltic Sea) or industrial (paper machine) waters. The steel types were those currently used for the construction of machines and installations involving large-scale use of water, such as the cooling systems of power plants, wet ends in the paper industry, and the food industry (Table 7). Steel finishings from delivery grade to 500 grit polished were used in the process equipment in these industries. Biofouling and microbially influenced corrosion (MIC) were investigated on steel types UNS S31600, S30400, S31254 and N08904 (Table 7).

3.2 Experimental set-ups used in this biofilm study

Biofouling and biocorrosion are major causes of downtime and maintenance costs in the paper industry and in power plants. Understanding the sequence of events leading to fouling and corrosion will help to design protocols for minimizing these problems.

Table 5 Cataloque of the methods used in this study

Method	Described in:
ATP content of bacteria	Paper II
In situ hybridization	Kolari et al 1998, This thesis
SEM analysis	Paper II, Väisänen et al 1998
TEM analysis	Paper I, Nurmiaho-Lassila et al 1990
EDS elemental analysis	Paper IV
CLSM analysis	Paper II and Paper III
Open circuit potential measurement	Paper I, Carpen et al 1995
Laboratory mesocosm	Paper I
Splash zone simulator	Paper IV, Carpen et al 1999
Flow cell for industrial applications	Paper III
Staining with fluorescent beads	Kolari et al 1998, Paper III
Staining with EtBr, syto 16, acridine orange, Live-Dead stains, concanavalin A	Kolari et al 1998
Scanning fluorometry	Paper III

Table 6 Properties of fluorescent stains used in the CLSM and epifluorescence studies of the stainless steel grown biofilms.

	Diameter or molecular weight	Fluorescence emission maxima	Concentration	Surface properties
Nucleic acid stains				
Ethidium bromide	394 g mol^{-1}	605 nm	100 µg ml^{-1}	log K_{ow} –0.38
Acridine orange (DNA)	302 g mol^{-1}	526 nm	100 µg ml^{-1}	log K_{ow} 1.24
SYTO™ 16	≈ 450 g mol^{-1}	518 nm	20 µM	log K_{ow} 1.48
SYTO™ 9 (LiveDead®)	≈ 450 g mol^{-1}	530 nm	3.34 µM	
Propidium iodide (LiveDead®)	668 g mol^{-1}	617 nm	20 µM	
Fluorescent beads for detecting porosity or surface properties				
Carboxylate-modified	0.02 µm±15.8%	605 nm	9 x 10^{12} beads ml^{-1}	hydrophilic
Aldehyde-sulfate-modified	0.029 µm±20.1%	515 nm	3 x 10^{12} beads ml^{-1}	hydrophobic
Miscellaneous stain for detecting glucose and mannose containing residues				
Concanavalin A, tetramethylrhodamine conjugated	104 000 g mol^{-1}	572 nm	200 µg ml^{-1}	hydrophilic
Fluorescent labels used in oligonucleotide probes				
Fluorescein		518 nm		
Cy 3		563 nm		
Oregon green		524 nm		

Behavior of light in fluorescence techniques: *Fluorescence is the result of a process that occurs in fluorophores or fluorescent dyes. A photon of energy (light) is supplied by an external source such as laser and absorbed by fluorophore. Due to this excitation the energy level of fluorophore is changed. When returning to original energy level the fluorophore emits a photon of energy (light). Due to energy dissipation during the excited-state lifetime, the energy of this emitted photon is lower i.e. longer wavelenght. The change in wavelenghts is called Stokes shift and is fundamental to fluorescent techniques (Haugland 1996).*

When two different wavelengt of light are emitted at the same spot the visible light is combination of the both. If same bacteria is hybridized both with fluorescein (green) and Cy3 (red) labelled probes the join signal is yellow. The same happends with acridin orange, joint signal of DNA (green) and RNA (red). If red, green and blue are joint we will see "normal" white light.

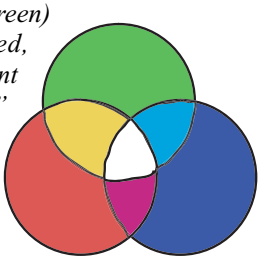


Table 7 Composition of the stainless steel types studies in this thesis.

Steel type	**Elements**	(%, w/w, elements other that Fe)					
UNS	C	Si	Mn	S	Cr	Ni	Mo	Cu
S31600	0.03	0.56	1.51	0.04	16.9	10.7	2.6	-
S31254	0.01	0.42	0.40	0.002	20.1	17.2	6.1	0.67
S30400	0.04	0.46	1.48	0.005	18.4	8.6	0.1	0.2
N08904	0.02	0.53	1.48	0.002	19.5	25.0	4.5	1.4

Baltic Sea water is used for cooling purposes in power plants located along the coast. For this reason, its capacity to form biofilms or to induce biocorrosion of stainless steels is not only of scientific, but also of economic importance. We set up a field study site in the vicinity of Vallisaari, near the Helsinki coastline. The field study equipment (details in Carpén et al 1995) was placed at the depth of 15 m, where the penetration of daylight is < 1 % (estimated based on the method described by Vähätalo 2000). This was done in order to reduce algal and other phototrophic growth on the steel coupons.

For measurements that could not be performed under field conditions, we built a large laboratory simulator of the Baltic Sea ecosystem, here called the mesocosm. It consisted of an illuminated chamber fed with Baltic Sea water from a 800 l container, and of a dark chamber from which the water was pumped into Robbin's devices (Fig 1 in Paper I). Hydraulic retention times in the light (1850 lux, 12 h day^{-1}, 100 l) and the dark (25 l) chambers of the mesocosm were adjusted to maintain a balance between the mineralization of organic carbon (dark reaction) and the primary production (light reaction). In this way the concentration of dissolved organic carbon was maintained at the natural level of Baltic Sea water, 4-7 mg of non-particulated (< 0.02 mm) organic C per litre. Stainless steel coupons were placed in modified Robbin's devices submerged in the water recycling pipe connecting the dark and the light compartments of the mesocosm. The mesocosm was fed with Baltic Sea water collected from the field study area. The composition of Baltic Sea water is given in Table 8. The Baltic Sea biofilms were grown for 4 to 8 weeks in the field and in the laboratory during all seasons of the year. The conditions in the laboratory mesocosm were similar to the natural microbial ecosystem of Baltic Sea water, except for the temperature (23ºC).

The paper making industry continues to be a heavy user of fresh water in Finland (275 million m^3 per year in 2001, personal communication Kari Luukko, Metsäteollisuus ry), in spite of the development in water-saving technologies such as the closure of mill water circulating systems. We investigated the biofouling of stainless steel surfaces inside the paper mill, as well as in a laboratory model of the machine splash area.

Biofilms were grown on coupons of stainless steel (diameter 25 mm) in flow cells (Fig 1 in Paper III) connected to the side flow of the spray water circuit of a paper machine. The main properties of the spray water are shown in Table 8. In the paper mill, spray water is used for cleaning the returning wire(s) of the paper machine. The spray water is filtered and contains almost no fibres.

A simulator was developed to study corrosion related to biological deposit formation in the splash areas of paper machines. It was modified from a test arrangement originally designed by Hakkarainen (1999) for studying the use of chlorine dioxide for the bleaching of pulp. The simulator contained stainless steel coupons, thermostated to 45ºC, and covered by sheets of pulp under a glass dome. The pulp sheets covering the coupon were continuously sprayed with simulated white water (Table 8) in order to mimick the situation in the paper machine. However, the NaCl concentration was much higher (0.5 to 1 g l^{-1}) than that in the "normal" white water of a paper machine (< 50 mg l^{-1}). The set up of the simulator is shown in Figure 1 of Paper IV. The simulator enabled determination of the mass balance between the feed, the deposits formed inside the simulator, and the effluent discharged from the simulator.

The formation of indigenous deposits on the steels was studied using coupons of

Table 8 Characteristics of the waters used as substrate for biofilm growth in this thesis.

	Baltic Sea water	Paper machine spray water	Simulated paper machine white water
pH	7.3-8.0	4.9-5.5	5.3
Temperature range ^{o}C	0-32	41-48	45
SO_4^{-2} mg l^{-1}	340-540	1100	950
Cl^- g l^{-1}	2.6-3	0.022	0.5-1.5**
TOC mg l^{-1}	4-7	270	400* (DOC)
Tot N µg l^{-1}	300-800		260*
Tot P µg l^{-1}	20-40		1600*

** Concentrations calculated from added amounts. Full recipe of simulated paper machine water is in M&M section of Paper IV.*
*** The high amount of chlorine was added to promote corrosion events.*

stainless steel (150 mm x150 mm) placed in the splash zone (≈40ºC) of the wire section in a paper machine. The exposed coupons were inspected by microscopy upon sampling.

3.3 In situ hybridization

Coupons of stainless steel with biofilm were rinsed with sterile water, air–dried and stored desiccated at room temperature. Table 9 describes the oligonucleotide probes used. The dry coupons were pretreated with ethanol: 30 % aqueous formaldehyde (90:10 v/v) for 5 min, rinsed with sterile water, and the excess water drained off (protocol modified from Braun-Howland et al 1992). The ethanol-formaldehyde treatment was used to reduce non-specific fluorescence (Amann et al 1992). The hybridization mixture used for probes EUB338, ALF1b and GAM42a contained 20-35 % formamide, 62.5 ng of each probe in 20 µl, 0.9 M NaCl, 20 mM Tris-HCl pH 7.2, and 0.01 % SDS (Manz et al 1992). Twenty microliters of this mixture were applied on the biofilm on a steel coupon and incubated for 2 h at 46ºC in a humidity chamber. Unbound probe was removed with 2 ml of washing solution: 20 mM Tris, 0.01% SDS, 5 mM EDTA and 40-180 mM NaCl. The coupons were then immersed in 50 ml of the washing solution at 48ºC for 20 min and rinsed briefly with sterile water. The hybridizations with SRB358 and DTM229 were conducted using SRB specific conditions as described by Zarda et al 1997.

Table 9 Oligonucleotide probes used for in situ hybridization of biofilms.

Probe	Sequence 5' – 3'	Target gene and *E.coli* numbering	Specific for	Label	% formamide (hybridization)	NaCl (mM) (washing)	Reference
EUB338	GCTGCCTCCCGTAGGAGT	16S (338-355)	domain *Bacteria*	Fluorescein	20	180	Manz et al 1992
ALF1b	CGTTCG(C/T)TCTGAGCCAG	16S (19-35)	alpha *Proteobacteria*	Cy3	20	180	Manz et al 1992
GAM42a	GCCTTCCCACATCGTTT	23S (1027-1043)	gamma *Proteobacteria*	Cy3	35	40	Manz et al 1992
SRB385	CGGCGTCGCTGCGTCAGG	16S (385-402)	delta *Proteobacteria*	Cy3	20	308	Amann et al 1992, Zarda et al 1997
DTM229	AATGGGACGCGGAX*CCAT	16S (229-247)	*Desulfotomaculum* genus specific	Oregon green	15	-	Hristova et al 2000

* X indicates that one of bases A, C, G or T is used.

4 Results and discussion

4.1 Properties of biofilms as displayed by scanning electron microscopy, SEM

4.1.1 Visualising the formation of biofilms on stainless steel in the Baltic Sea water and in the paper machine wet end

Biofilms formed in the Baltic Sea water mesocosm (Fig 3 in Paper I) consisted of clusters of different kinds of bacteria on the ennobled stainless steel surface: bacteria with an appearance similar to *Seliberia* (spiral), *Caulobacter* (stalked) or *Hyphomicrobium* (cocci with tail), as well as small rod-shaped bacteria (≤ 1 μm in length, Fig 8a) (Boone et al 2001). We regularly observed diatoms on the steel surfaces in the immediate vicinity of clusters of biofilm bacteria, and sometimes inside the cluster (Fig 11, Fig 6 in Paper I).

A conditioning layer is defined in the literature as the film formed on solid substratum immediately after it is immersed, before any bacteria become attached (Costerton and Lappin-Scott 1995). Figure 8b shows an example of a Baltic Sea water biofilm that had grown for 22 days in the mesocosm (≈23ºC). A thin (< 0.5 μm) film covering the attached bacteria is visible. This film also covers the steel surface in areas where there are no attached microbes (bottom left corner of the figure). The film may or may not be what has been described in the literature as a "conditioning layer". Figure 8b shows several bacterial cells overlaid by a thin film, indicating that the film must have been generated after, and not before, the attachment of the primary colonizing bacteria. The film may originate from the excreta of surface-attached bacteria or from the deposition of dissolved or colloidal matter in the surrounding water (Kepkay et al 1993, Schuster and Herndl 1995, Middelboe et al 1995), part of which may be produced by the activity of the adhered bacteria (Wingender et al 1999).

Bacteria are known to excrete polymeric organic matter. Such polymers may play a role in the generation of biofilm clusters, or help individual bacteria to adhere. The production of exocellular polymeric substances is limited by the available energy. There should be a good reason for excreting exopolymers, e.g. for protection against grazing biota. Evidence obtained by Fourier-transformed infrared spectroscopy of organic films in seawater indicates that glycoproteins, proteins and possibly humic acids may be involved (Korber et al 1995). Humic substances are abundant in Baltic Sea water. This may in part explain the origin of the organic films visible in Fig 8. Baltic Sea water is cold (0 to 17ºC, depending on the season) and oligotrophic. *Seliberia, Caulobacter* and *Hyphomicrobium* are genera physiologically adapted to live in such environments (Corpe and Jensen 1996, Stahl et al 1992, Lomans et al 1999). All three are oligotrophic chemo-organotrophs.

The warm (> 40ºC), mildly acid paper machine environment is very different from that of the Baltic Sea water, which is neutral in pH and colder. The total organic carbon content of paper machine spray water is also 50 times higher than that in Baltic Sea water (Table 8), and would therefore be expected to favour bacteria different from those in the Baltic Sea. Biofilms grown for 5 to 7 d in paper machine water flow in the mill consisted of bacteria growing in clusters (Fig 9). No fungi or other eukaryotes were seen in the biofilm. The bacteria were uniform in shape and size within each biofilm cluster, but the shapes and/or sizes of the bacteria varied from cluster to cluster (Fig 10). In the paper machine biofilms we observed only three morphologically distinguishable types of

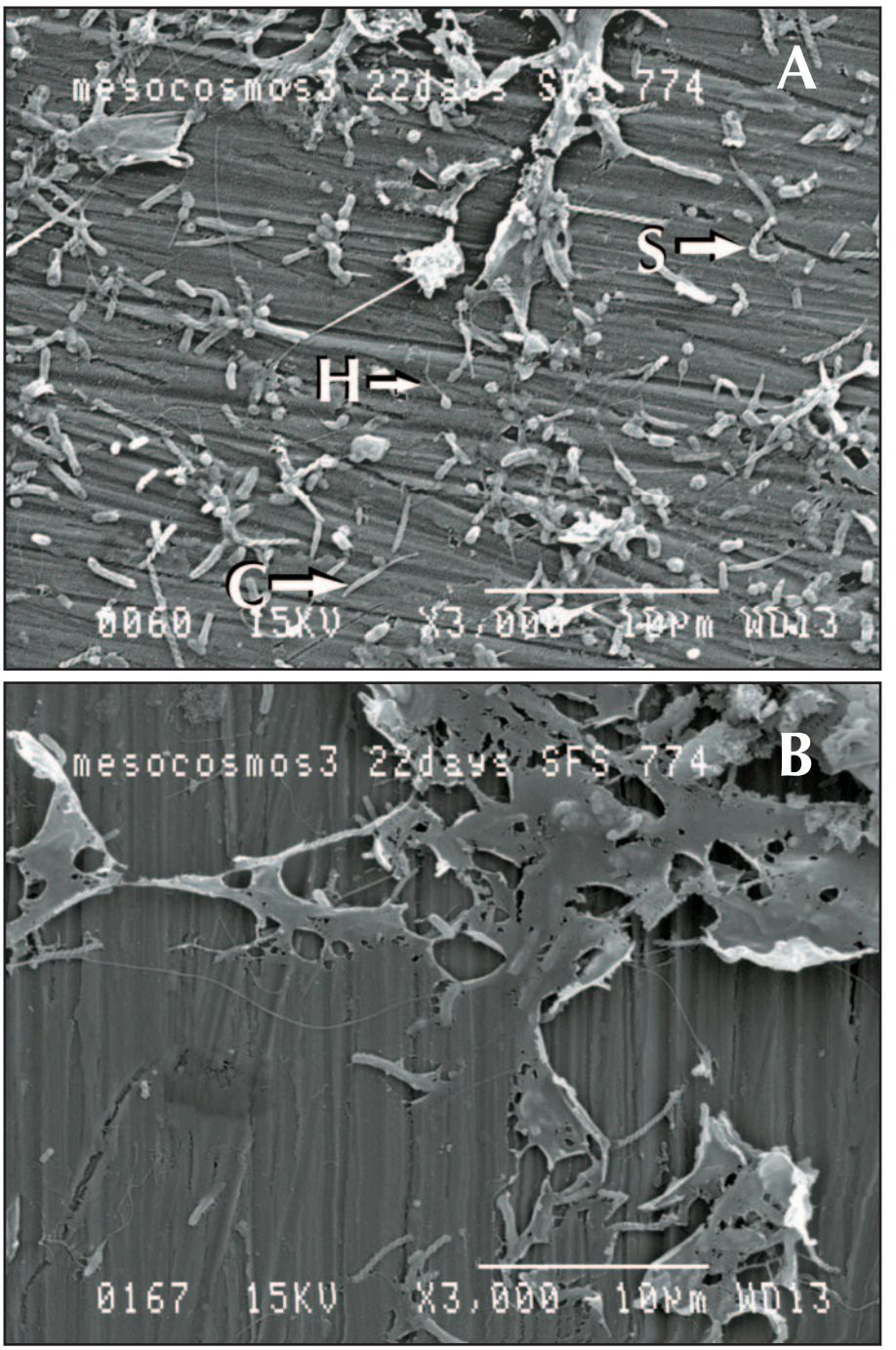


Figure 8. Scanning electron micrographs of biofilm formed on stainless steel (UNS N08904) exposed to Baltic Sea water for 22 d in the laboratory mesocosm (≈23º C). A) Bacteria morphologically recognisable as; Seliberia (s), Caulobacter (c) and Hyphomicrobium (h). Horizontal grooves originating from steel polishing are visible. B) A thin "conditioning-like" film covering large areas of the steel surface.

cell: uniformly coccoid (Fig 2 in Paper III), filaments with an identical appearance (≈ 50 µm in length, Fig 4 in Paper III), and rods of varying length (1 µm to 6 µm, Fig 5 in Paper III). Several long, rod-shaped bacteria have been earlier reported from the paper machine wet end: the novel genus *Thermomonas* (Busse et al 2002), species of *Burkholderia (B. cepacia) Bacillus, Paenibacillus, Enterobacter* and *Meiothermus* (Väisänen et al 1989a, 1989b, 1994 and 1998, Kolari et al 2001, Suominen et al 2002). Short rods detected in the paper machine wet end area have been identified as species of *Sphingomonas, Acinetobacter, Klebsiella* and *Brevibacterium* (Väisänen et al 1994 and 1998). The cocci frequently found in the paper machine wet end include the genera *Deinococcus, Cellulomonas* and *Micrococcus* (Väisänen et al 1998, Kolari et al 2001).

4.1.2 Biofouling of stainless steel surfaces exposed in the Baltic Sea and in a paper machine

The accumulation of material on non-living surfaces, whether formed by bacteria or by chemical and/or physical processes, is called fouling. Fouling decreases the flow rates in pipelines, decreases the efficiency of heat transfer in heat exchangers (Flemming 1996), causes clogging, and deteriorates the hygienic quality of the paper product (Väisänen 1989a and 1989b, Pirttijärvi 2000). Biofouling thus results in a need for cleaning, thereby increasing costs.

The Baltic Sea biofilms frequently contained cyanobacteria (Fig 11). Diatoms were more frequent on the steel surfaces exposed in the illuminated chamber of the laboratory mesocosm than on the coupons placed in the Robbin's devices in the dark compartment (Fig 17, Figs 6-8 in Paper II).

In the paper machine biofilms, wood fibres and pulping fines were the major non-microbial components (Fig 12 in Paper III). Figure 12 shows a biofilm (5 days old) formed *in situ* in the spray water circuit of a paper machine. Spray water represents one of the cleanest types of water in a paper machine, and is low in fibre and contains primarily colloidal and finely dispersed solids. Nevertheless, the figure shows a steel surface covered mostly by substances other than bacteria. The bacteria were found to be present in the first layer of the biofilm (Fig 7 in Paper III). The bacteria were no longer visible by SEM during the later stages of biofilm growth. The proportion of bacteria decreased as the age of the biofilm increased (Fig 12 in Paper III). The ratio between bacteria and other components in the biofilms did not remain constant over the seasons. During spring and autumn the nutrient content of natural waters is higher compared to that in other seasons (Pesonen et al 1995). Bacteria use the nutrients in the incoming water as growth substrates.

Conclusions on biofilm formation as observed by SEM

The scanning electron microscopic evidence in our study shows that the biofilm bacteria in the two environments, i.e. the Baltic Sea and a paper machine, were morphologically different. The differences were presumably related to the different selective conditions in these environments. Other types of material were primarily attached on top of the thin layer of primary-attaching bacteria in both the Baltic Sea water and paper mill biofilms.

4.1.3 Corrosion- related microbiologial events on stainless steel in Baltic Sea water: factors influencing ennoblement

Our aim was to identify the environmental or microbiological factors influencing the development of ennoblement, and thereby the risk of corrosion of the steel during the growth of biofilms.

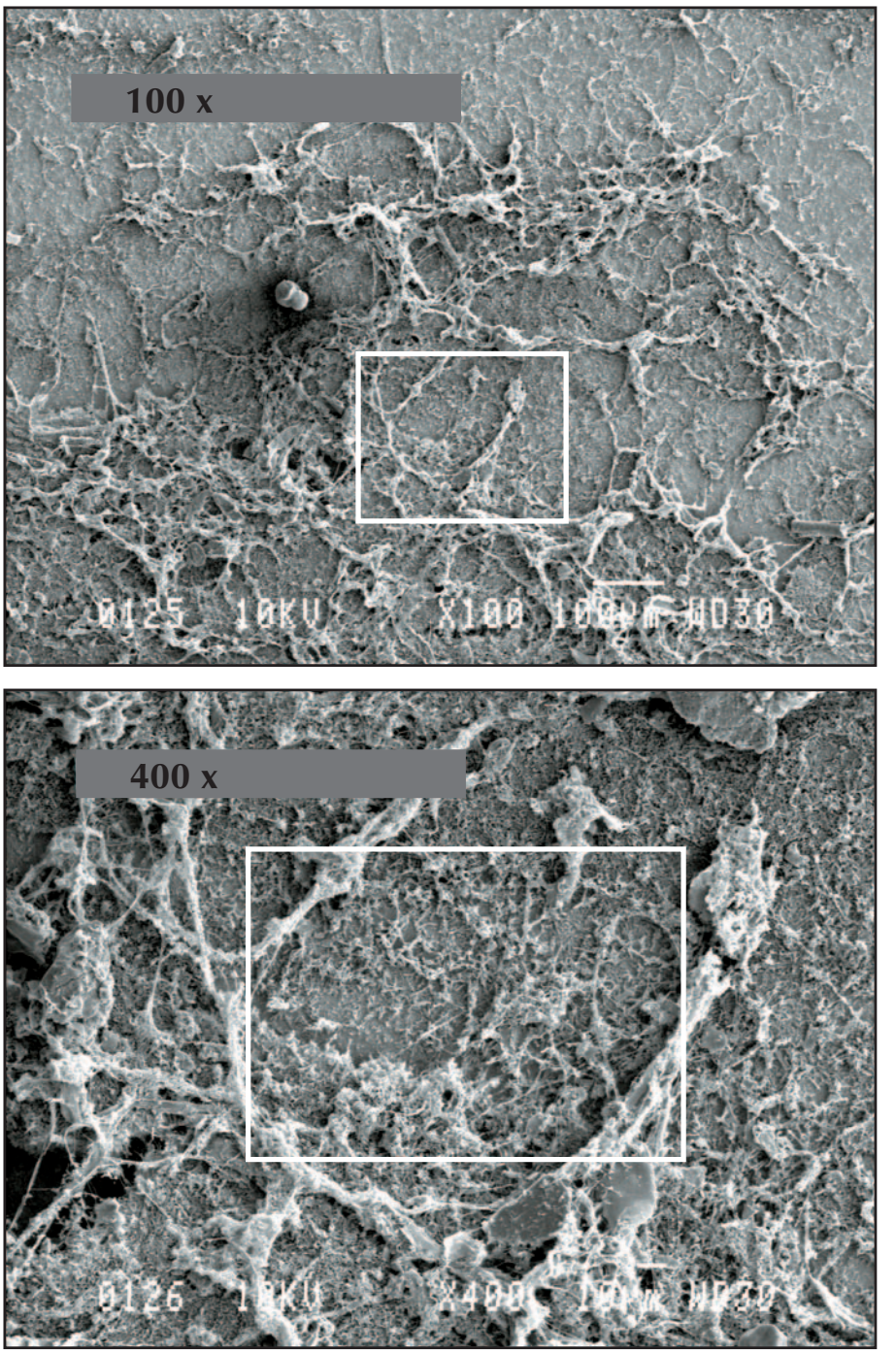
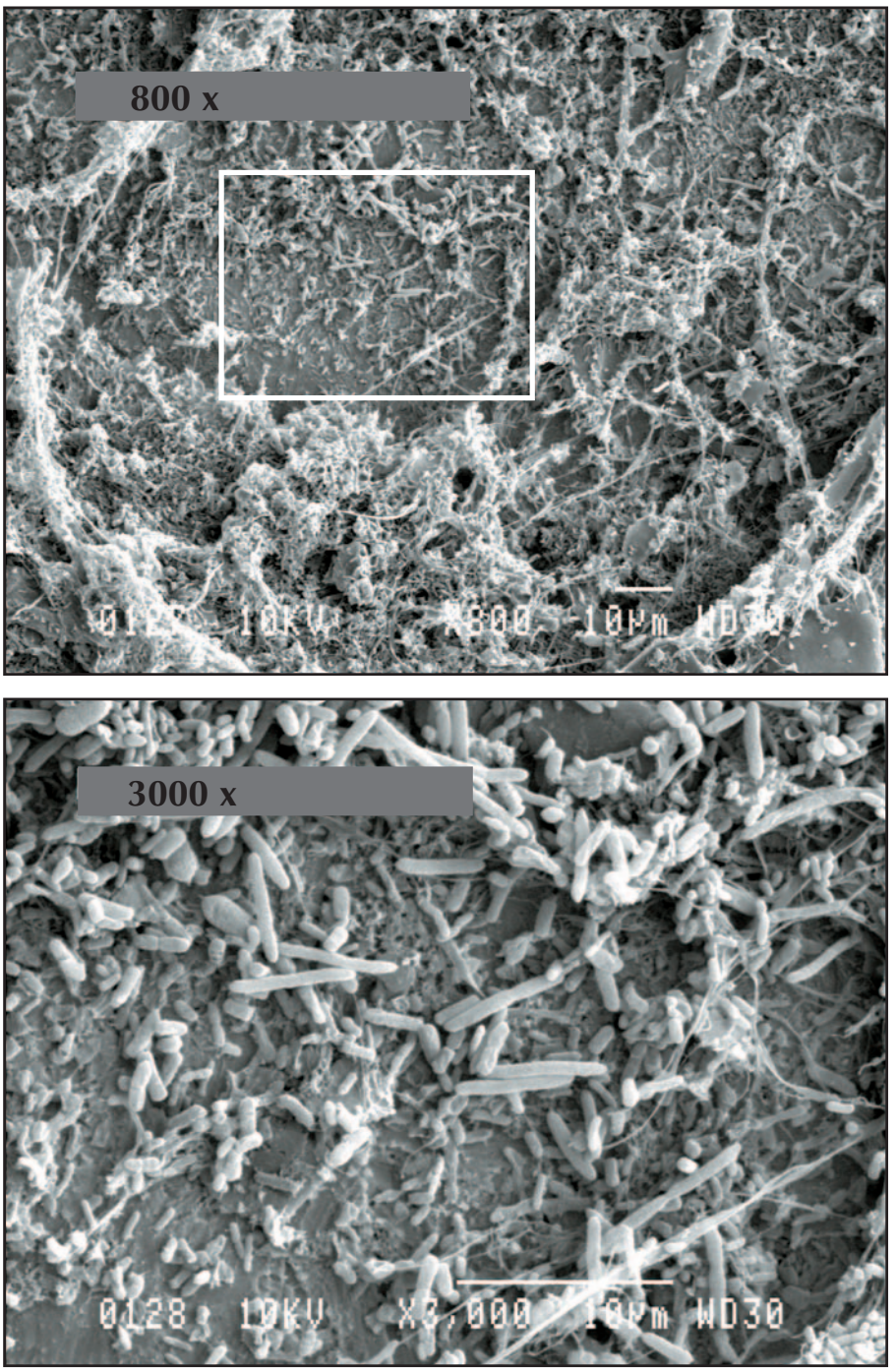


Figure 9. Scanning electron micrographs (SEM) of biofilm formed on stainless steel (UNS S31600) during 5 days of exposure to paper machine spray water (≈45º C) flow in the mill. The magnifications are 100 x, 400 x, 800 x and 3000 x. The box shows the area used for the zoom-up. The low magnification micrograph (100 x) shows the web like structure of the biofilm.



The 400 x to 3000 x magnifications show differently sized rod shaped bacteria (2-7 µm). SEM technique requires the samples to be fixed (glutaraldehyde) and fully dried before the image is taken. The desiccation may cause the microbes to shrink down to 1/3 or 1/2 of their volume compared to living cells.

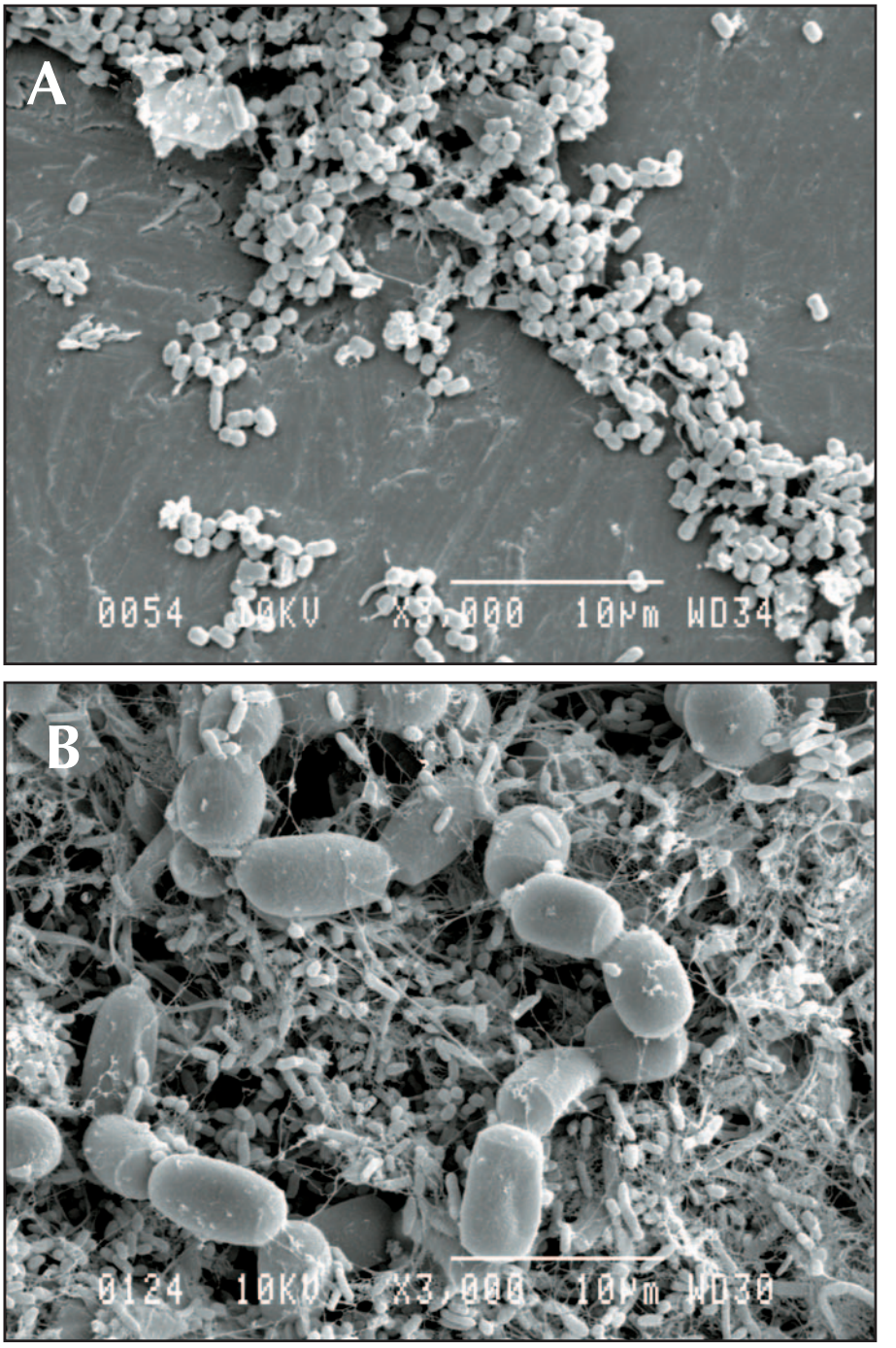


Figure 10. Scanning electron micrographs of 2 different biofilm clusters formed on a coupon of stainless steel (UNS S31600) exposed to paper machine spray water flow for 5 days of in the mill. Panel A shows biofilm aggregates containing bacteria with uniform cocci like appearance. Panel B shows rod shaped bacteria of 2 to 1 µm in length and large cyanobacteria like cells (3 µm x 8 µm).

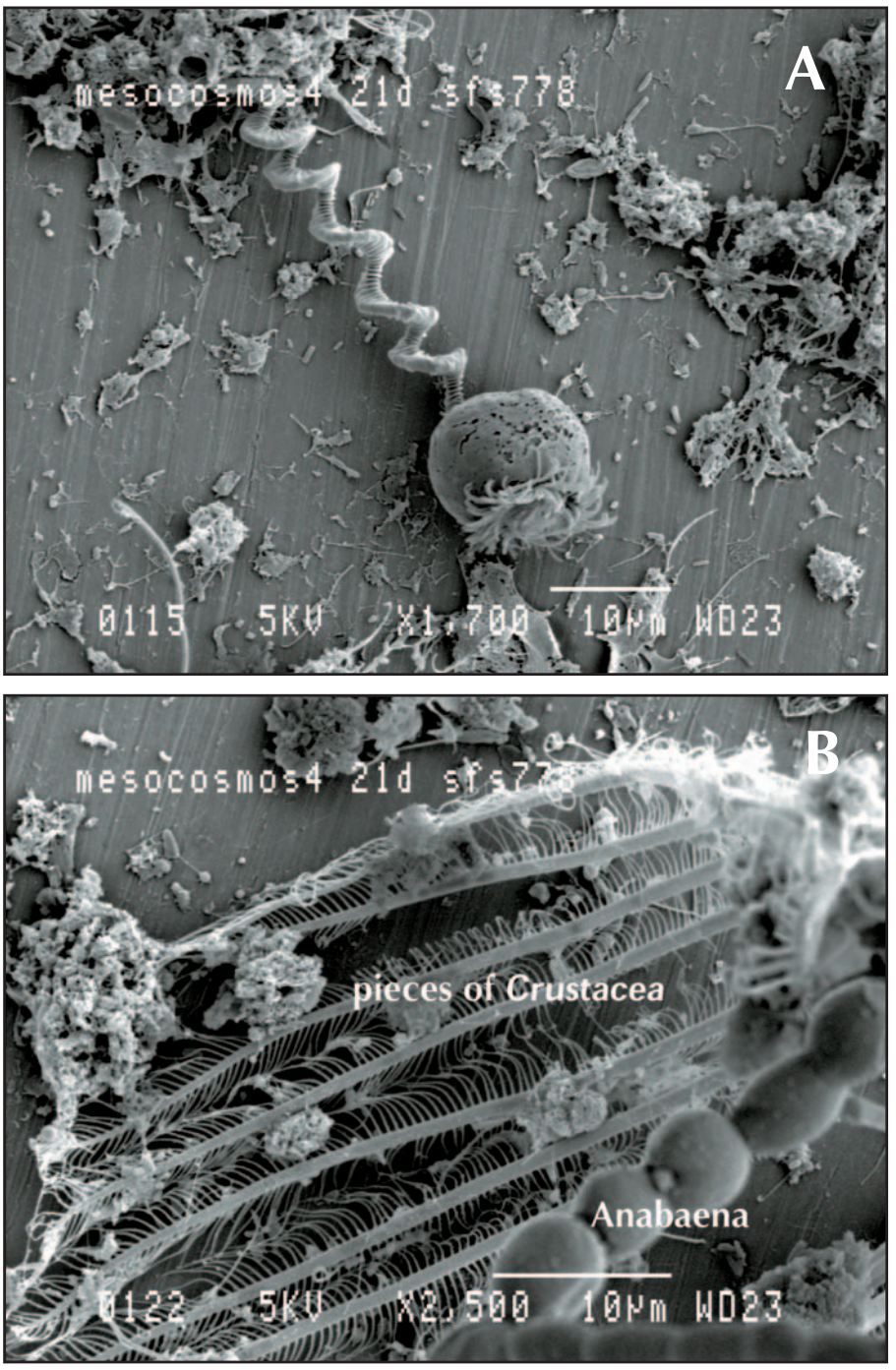


Figure 11. Scanning electron micrograph of biofilm formed on stainless steel (UNS S31254) exposed to Baltic Sea water for 22 d in the laboratory mesocosm (≈23ºC). Panel A shows a Vorticella like protozoan grazing in the biofilm. Panel B shows a chain of cells resembling the cyanobacterium Anabaena and pieces of Crustaceans .

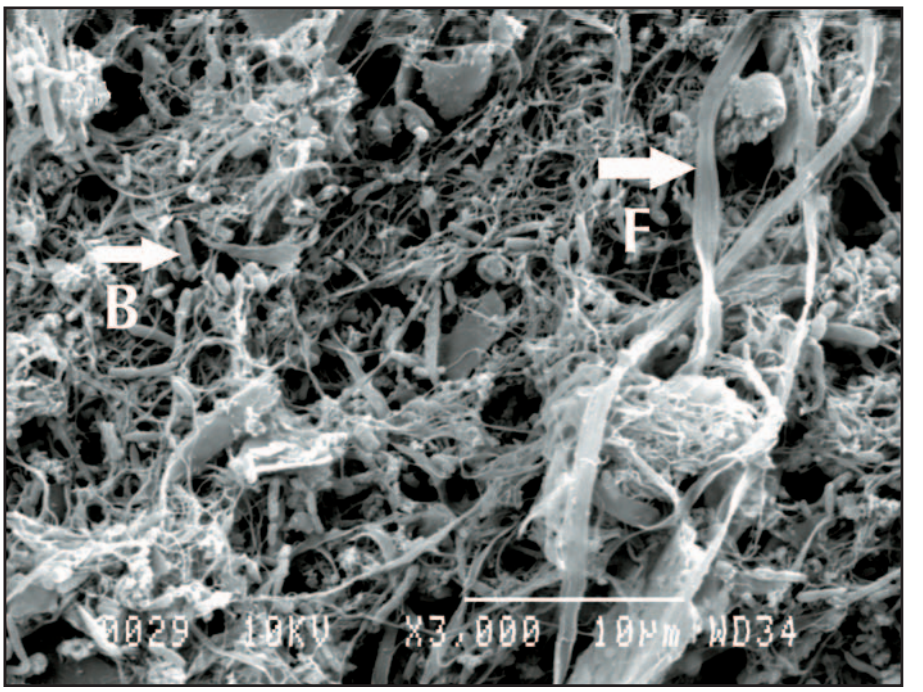


Figure 12. Scanning electron micrographs of a biofilm formed on stainless steel (UNS S31600) during exposure to paper machine spray water for 5 days in the mill. Here the biofouled steel is covered by fines and fibers (F) originating from pulping. Bacteria (B) are minor constituents in this biofilm.

Ennoblement of the steel was inhibited by doubling the concentrations of ammonium and phosphate (increase of 778 mg N l^{-1} and of 33 mg P l^{-1}) in the Baltic Sea water in the laboratory mesocosm under otherwise favourable conditions. No ennoblement occurred in the steel coupons in the laboratory ecosystem when the feed was Baltic Sea water enriched by an accidental, month-long discharge of treated sewage, which raised the total nitrogen content in the seawater two fold over the natural level of 300-800 mg N l^{-1}, but caused no changes in the phosphorus level. The biofilm that grew in this water differed from the biofilm formed in the more oligotrophic water in that it was easily detached from the steel surface and had no compact microbial clusters (Fig 8 in Paper II).

The conditions that allowed formation of ennobling biofilms were a flow rate of 10-35 mm s^{-1} and low nutrient concentrations (Table 1 in Paper II). When the flow rate was low, i.e. < 10 mm s^{-1}, loosely attached, fluffy biofilms grew on the steel surfaces. These biofilms had a large surface coverage but did not increase the open circuit potential of the underlying steel (Tables 1, 2 in Paper II).

In the highly structured biofilm, the bacterial clusters that co-emerged with ennoblement of the steel were separated from each other (clear-edged structure). A high flow rate favoured the formation of a biofilm in which the microbial cluster was strongly attached to the substratum (the last paragraph in the Results Section of Paper I, and the last paragraph in the Results

Section of Paper II). A compact biofilm cluster may prevent oxygen penetrating into the steel surface below the biofilm. As a consequence, the flow of electrons may become channelled towards iron (Fe^{3+}–>Fe^{2+} [E_0´+770 mV], O_2 –>H_2O [E_0´+820 mV], Nealson and Myers 1992). These structural properties will contribute to the formation of differentiated aeration cells, leading to corrosive events (Watkins Borenstein 1994).

During the winter season the open circuit potentials of the stainless steel coupons immersed in the Baltic Sea increased from -200 mV_{sce} to 250 mV_{sce} in 50 days at 0°C. Coupons of the same steel showed no ennoblement at all within 38 days when immersed in Baltic Sea water thermostated at 32 to 36 °C in the laboratory mesocosm. When the temperature of the water where these coupons were immersed was cooled to 26°C, ennoblement occurred within 20 days. This shows the existence of an upper temperature threshold of ≤ 32°C for the ennoblement of stainless steel in Baltic Sea water (Fig 2 in Paper II). The temperature dependence of ennoblement indicates a connection to temperature-limited biology. Since the temperature in the Baltic Sea is typically between 0 to 15°C (Pesonen et al 1995), an increase of 20°C may block the growth of the indigenous microorganisms involved in ennoblement.

Microscopic inspection showed that the steel surface was only partially covered with biofilm at the time when ennoblement occurred in the laboratory mesocosm with Baltic Sea water (Fig 4 in Paper I, Fig 6 in Paper II). Partial coverage indicates that dissolved oxygen had free access to the uncovered parts of the metal surface during ennoblement. The ennoblement of stainless steel may thus require partial, but not full coverage, of the steel surface by a compact biofilm that insulates patches of the steel surface against the diffusion of oxygen. Ennoblement may be associated with the co-existence of patches with different access to oxygen. The patchy, electrically insulating biofilm may thus form the basis for the onset of corrosion.

We measured the content of ATP in biofilms on the steel surface, as well as the total dry weight, in order to determine whether there is a threshold for the density or the surface coverage of biofilm before ennoblement can occur. If this was the case, such a parameter could be used for predicting the occurrence of corrosive events.
Our results showed that neither the amount of ATP nor that of dry matter in Baltic Sea biofilms predicted the onset of ennoblement and thus a potential risk for corrosion of the steel (Table 2 in Paper II). The results were compatible with the view that corrosive events are related to a threshold in the surface coverage of a specific type of biofilm rather than to the overall biomass.

4.1.4 Biological events linked to the corrosion of stainless steel in a paper machine

Only a few studies have been carried out on the microbial communities in the wet end and the splash area of paper machines (Väisänen et al 1989a, 1989b, 1994 and 1998, Pirttijärvi et al 2000, Lindberg et al 2001b, Kolari et al 2001, Busse et al 2002), or on microbially induced corrosion in these sites (Vestola and Korhonen 1976, Soimajärvi et al 1978). The wet end area of a paper machine frequently suffers from corrosion (Carpén et al 2001a, Thorpe 1985). We found high concentrations of soluble oxalates plus oxalic acid (max 2300 mg kg^{-1}), and of thiosulphate (max 16000 mg kg^{-1}), in wet end deposits. Thiosulphate is known to be corrosive to stainless steel (Garner and Newman 1991, Laitinen 1999). The oxalic acid present under biofilms and other deposits on steel surfaces may lower the pH down to 2, thereby contributing to corrosion (Anonymous 1994). The process

water contained practically none of these compounds (Table 2 in Paper IV): < 5 mg thiosulphate kg^{-1} wet wt. and < 2 mg oxalate kg^{-1}.

A laboratory simulator was designed to investigate the origins of oxalate and thiosulphate in the wet end deposits of paper machines (Paper IV, Carpen et al 1999, 2000, 2001abcd). In this setup, called "the splash area simulator", stainless steel can be exposed to process water under conditions simulating those in the splash areas of the paper machine wet end.

The splash area simulator was used to investigate the events leading to the formation of corrosive deposits in the paper machine. In the simulator, a coupon of stainless steel was overlaid with sheets of pulp preinoculated with strains of *Bacillus subtilis, Desulfovibrio desulfuricans* and *Desulfotomaculum thermoacetoxidans,* and with an unidentified SRB culture enriched from paper machine white water (Paper IV, M&M). The simulator was fed with synthetic white water (Table 8), spiked with varying amounts of chlorine, to speed up corrosion. The chemical composition of the water leaving the simulator was measured. Thiosulphate (80 mg l^{-1}) and oxalate (70 mg l^{-1}) appeared in the effluent leaving the simulator when the simulator had been operating for two weeks. As these ions were not present (thiosulphate < 10 mg l^{-1}, oxalate < 5 mg l^{-1}) in the feed, thiosulphate and oxalate must have been formed *de novo* inside the simulator.

The open circuit potentials of the steel coupons placed in the paper machine simulator did not increase, indicating that no ennoblement occurred. After 28 days of operation of the simulator, the stainless steel coupons were removed and inspected by SEM for pitting. Stainless steel S30400 had become pitted over the whole surface underneath the pulp sheet during exposure. Stainless steel S31600 showed pits only in the peripheral area, i.e. at the junction of the pulp sheets and the glass dome covering the sheets. Microbial cells of 2-3 μm in size were found in the pits (Fig 13a) formed on steel S30400 during exposure in the simulator. These microbes may have contributed to the corrosion of the steel, or they may simply have sought shelter in the already formed corrosion pits. The pits were found in the preinoculated simulators only. Sulphate reducing bacteria were detected on steel surfaces in the preinoculated simulators (3rd chapter of Results in Paper IV).

Using SEM-EDS we found crystals on the steel surfaces exposed in the simulator where the pulp sheets had been preinoculated. The crystals had an elemental composition characteristic of calcium oxalate, calcium carbonate and calcium sulphate (Fig 13bcd). Crystallized calcium oxalate was also found in the deposits collected from the interior walls of a pulp slurry storage tank of a paper machine. Calcium oxalate is insoluble in water. If such crystals enter the wet end area of a machine, the pick-up felt and the wire may become clogged.

With SEM-EDS we detected deposits with a high silicon content all over the steel surfaces that had been exposed in the preinoculated laboratory simulator. Stoecker and Pope (1993) and Otero et al (1997) may have observed a similar phenomenon on steel exposed to a cooling water system using seawater. They suggested that the accumulation of silicon-containing materials on the stainless steel was linked to microbially induced corrosion. The same authors also found that bacteria belonging to the sulphate-reducing genus *Desulfovibrio* and the sulphide-oxidizing genus *Thiocapsa* were present during the microbially induced corrosion events in the coastal sea water. Our splash

area simulators were inoculated with *Desulfovibrio desulfuricans* (DSM 642), *Desulfotomaculum thermoacetoxidans* (DSM 5813) and a mixture of unidentified sulphate-reducing bacteria from the paper machine. Metabolism similar to that noted by Stoecker and Pope (1993) and Otero et al (1997) may have also taken place in the splash area simulator. According to Berthelin (1983) large amounts of complexing agents, like oxalic acid, can promote the solubilization of mineral elements (Si, Al, Fe, Mn, Ca, K) from granite rocks. In our system the silicon-containing deposits may have originated from the etching of silicon-containing steel (Table 7) in the aggressively acid conditions generated by the large amounts of oxalic acid (Table 2 in Paper IV). Microbiological transformations of silicon compounds were reviewed by Krumbein (1983).

Silicon compounds are excellent electrical insulators. Therefore silicon-containing layers may lead to the development of differential charges on the stainless steel, strengthening the theory that partial coverage by insulating material may be involved. Coverage of the steel surface by microorganisms or other material to the extent that it changes the access of oxygen to the steel surface, leads to a differential aeration cell. Formation of differential aeration cells is considered to be one of the major events in microbially influenced corrosion (Heitz 1996).

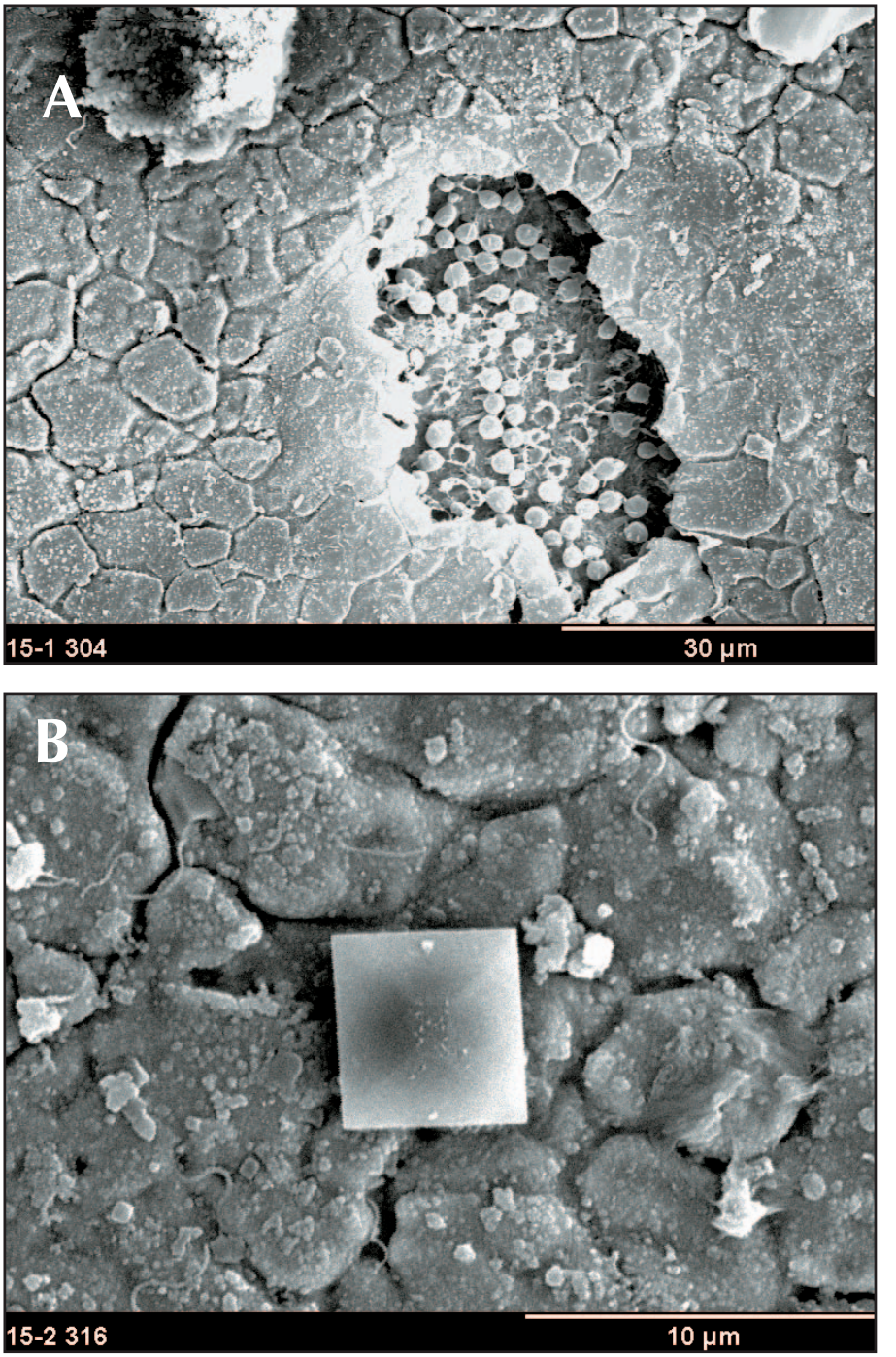


Figure 13. Scanning electron micrographs of stainless steel coupons (UNS S31600) pitted after being exposed to simulated paper machine process water in a splash area simulator operated at ≈45ºC for 28 d. A) Yeast-sized microbial cells are visible covering a pit. B) The EDS analysis revealed that this cubic crystal consisted of calcium and carbon, most likely of calcium carbonate or calcium oxalate.

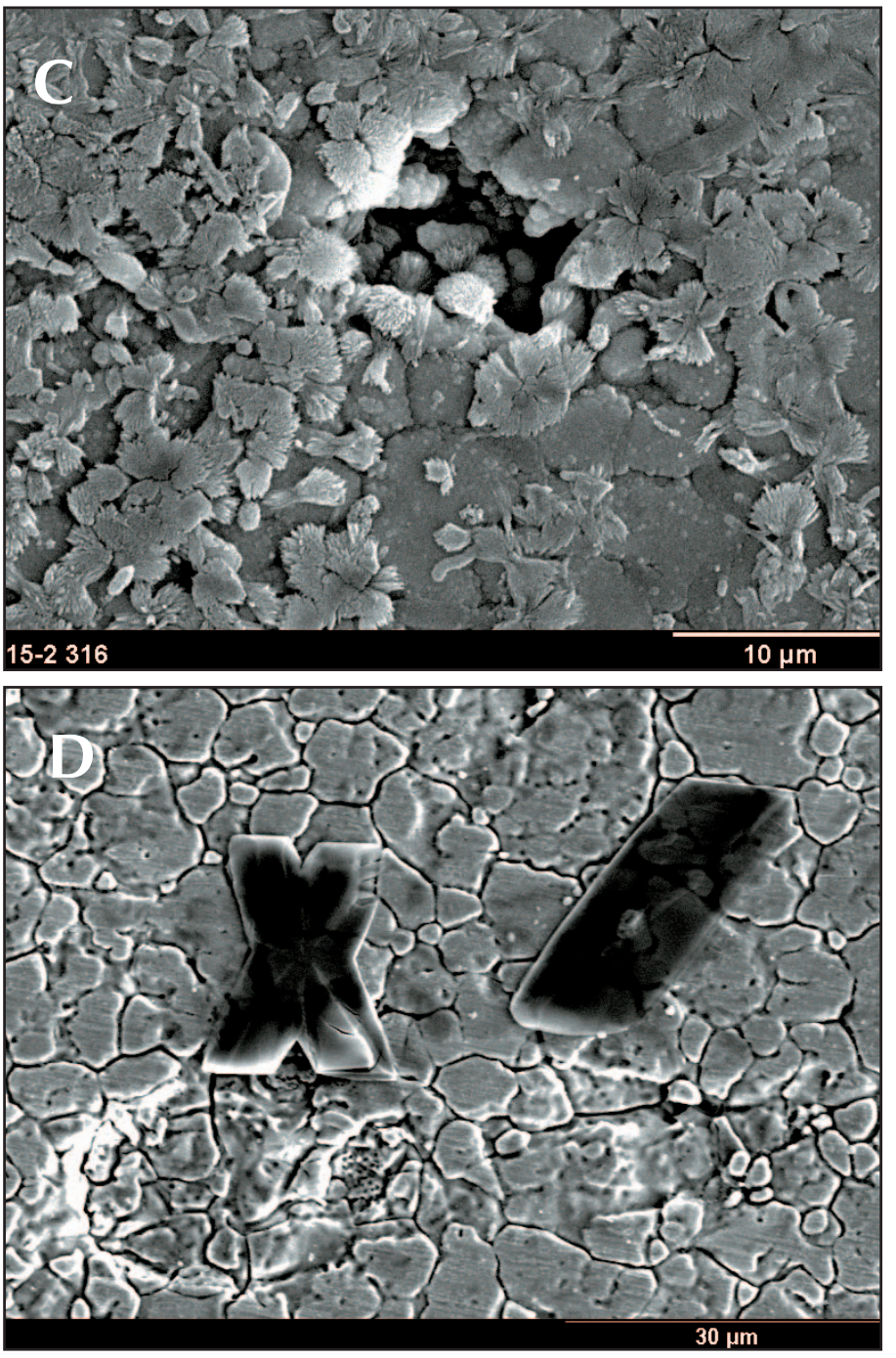


Figure 13 C) An example of an area where crystals covered the steel surface and the crevices formed in the steel. D) Crystals with x-form resembled calcium sulphate in elemental composition based on EDS analysis.

4.2 Properties of biofilms on stainless steels as displayed by confocal laser scanning microscopy (CLSM)

4.2.1 *In situ* hybridization in a study of biofilm forming bacteria

Fluorescent *in situ* hybridization with labelled oligonucleotide probes was used to obtain information on the taxons constituting the microbial biofilms generated in the spray water area of a paper machine and during the process of ennoblement of stainless steel in seawater.

We used eubacterial oligonucleotide probe EUB338 to detect the presence of bacteria and to test the success of the applied hybridization procedure. The hybridization protocols reported in the literature are for biofilms on the surfaces of glass or plastic. Stainless steel grown biofilms have not been investigated by FISH prior to this study. We found that EUB338 hybridized most of the bacteria (green fluorescence, fluorescein) in the Baltic Sea biofilms (Fig 14b), proving that the chosen hybridization protocol was operative. The alpha *Proteobacteria*-targeted probe ALF1b (red, Cy3) was used together with probe EUB338 (green) in Figures 14b and 15. The two probes hybridized into separate bacterial clusters in the biofilm. This is noteworthy, because the alpha *Proteobacteria* group should form a part of the domain bacteria to which EUB338 is targeted. The double-labelled bacteria would have fluoresced yellow (joint green and red). 11.8% of the bacterial 16S rRNA sequences in the databases matched perfectly with the ALF1b probe, while they exhibited 1 to 2 mismatches with the EUB338 probe (Kolari et al 1998). The red fluorescence is explained if the bacteria in that cluster belong to the 11.8 % mismatching with EUB338. The gamma group of *Proteobacteria* was searched for using the GAM42a probe (red fluorescence, Cy3) together with the EUB338 probe (green). The results showed that only a minority(< 15 %) of the biofilm-forming bacteria in the Baltic Sea biofilms were members of the gamma group of *Proteobacteria* matching with GAM42a (Fig 14a). This is less than the score for ALF1b, which hybridized with 25-30 % of the biofilm bacteria, indicating they were members of the alfa group of *Proteobacteria*.

Based on the results, above we presently conclude that the majority of the ennobled stainless steel biofilm-forming bacteria in the Baltic Sea were recognized by the EUB388 probe, but not by the alpha or the gamma proteobacterial probes. Therefore the bulk of the biofilm bacteria may represent eubacteria other than the alpha or gamma *Proteobacteria*. SEM micrographs of the Baltic Sea biofilms (Fig 8) showed the presence of C*aulobacter, Hyphomicrobium* and *Seliberia*-like bacteria, all of which belong to alpha *Proteobacteria.* Based on the sequence data alone, the *Caulobacter* should have hybridized with both probes (EUB338 and ALF1b), whereas *Hyphomicrobium* matched with EUB338 only and *Seliberia* did not match with EUB338 or with ALF1b.

Paper machine splash area biofilms from the mill were hybridized to the EUB388 probe (fluorescein, green) targeted to the domain bacteria, and to the SRB385 probe (red, Cy3) targeted to the sulphate-reducing delta subclass of *Proteobacteria*. Bacteria with a positive response to EUB388 (green, fluorescence) were detected, but if the red fluorescence expected from SRB385 was present at all it was masked by the strong autofluorescence of the paper machine fines (Fig 16b).

Probes for the gram-positive genus *Desulfotomaculum* DTM229 (oregon green) and for the sulphate-reducing delta *Proteobacteria* SRB385 (red, Cy3) were used to determine the presence of bacterial

groups with a metabolic capacity to reduce sulphate into sulphur-containing ions of lower oxidation states. Paper machine water contains 0.5 to 1 g of $SO_4^{2-}l^{-1}$ (Table 8), and sulphate is the substrate for sulphate-reducing bacteria. Their products, reduced sulphur-containing ions, may cause corrosion of stainless steel (Hamilton and Lee 1995). The splash area biofilms, formed on stainless steel in the paper machine, showed a faint binding by DTM229 to large sized bacteria (Fig 16a). No red fluorescing cells were detected, even though the hybridization conditions were chosen to favour SRB385 (Table 9).

Oligonucleotide probes EUB338 and ALF1b belong to fluorescence brightness group III described by Fuchs et al (1998), based on the secondary structure of rRNA. This means that they fluoresce with an intensity of 44 to 58% of the intensity exhibited by the brightest probe, Eco148L. The fluorescence intensities of EUB338 and ALF1a are therefore expectedly two times higher than that of the group IV probes SRB385 (≈39%) and DTM229 (≈26%). This may explain the observed differences in the signal detection of DTM229 versus EUB338 (Fig 16).

The result indicates that delta proteobacteria were not major biofilm constituents in the splash area of the paper machine, and that the genus *Desulfotomaculum* was present but not abundant. The Baltic Sea water biofilms were more readily analysable than the paper machine biofilms using hybridization methods, due to less interference by autofluorescencing substances. In cases where autofluorescence was observed in the Baltic Sea water biofilm it was of a defined shape (diatoms) and therefore easily identified. The large amounts of autofluorescencing papermaking fines in the biofilms disturbed detailed, fluorescence-based detection systems like *in situ* hybridisation. The presence of soluble and insoluble salts (Figs 13bcd, Table 2 in Paper IV) are also likely to disturb the hybridization procedure.

4.2.2 Use of autofuorescence and specific fluorescent stains for analysing the biofilms formed on stainless steel

The algae, especially silicon-containing diatoms, present in Baltic Sea biofilms have strong red autofluorescence. One or two diatoms per microscopic field (200 µm x 200 µm) were regularly observed on the surface of the stainless steel in these biofilms. Diatoms were useful for the interpretation of other CLSM results because they served as indicators of the laser beam penetration through the biofilm (Fig 6 in Paper I, Fig 7 in Paper II).

We used tetramethylrhodamine conjugated concanavalin A (conA) as an indicator of the presence of glucose- or mannose-containing polysaccharides in the paper machine biofilms. It stained yeast-sized cells, but not as successfully as the smaller (Ø 1 µm) biofilm-forming bacteria (Fig 3 in Paper III). The conA conjugate stained the amorphous fines of paper machine slimes, most probably due to the presence of mannose and glucose in cellulose and hemicellulose, respectively. Yeast-sized cells were found (Fig 13a) on steel coupons left for 28 days in the paper machine splash area simulator. Their cell size and shape was similar to the yeast-sized cells stained with con A (Fig 3 in Paper III).

4.2.3 Properties of the biofilm clusters grown on stainless steel

Knowledge of biofilm porosity is important for the planning of trategies for disinfection and for the removal of biofilms. If the biofilm cluster remains porous while growing in height/thickness, cleaning solutions may penetrate to the roots of the

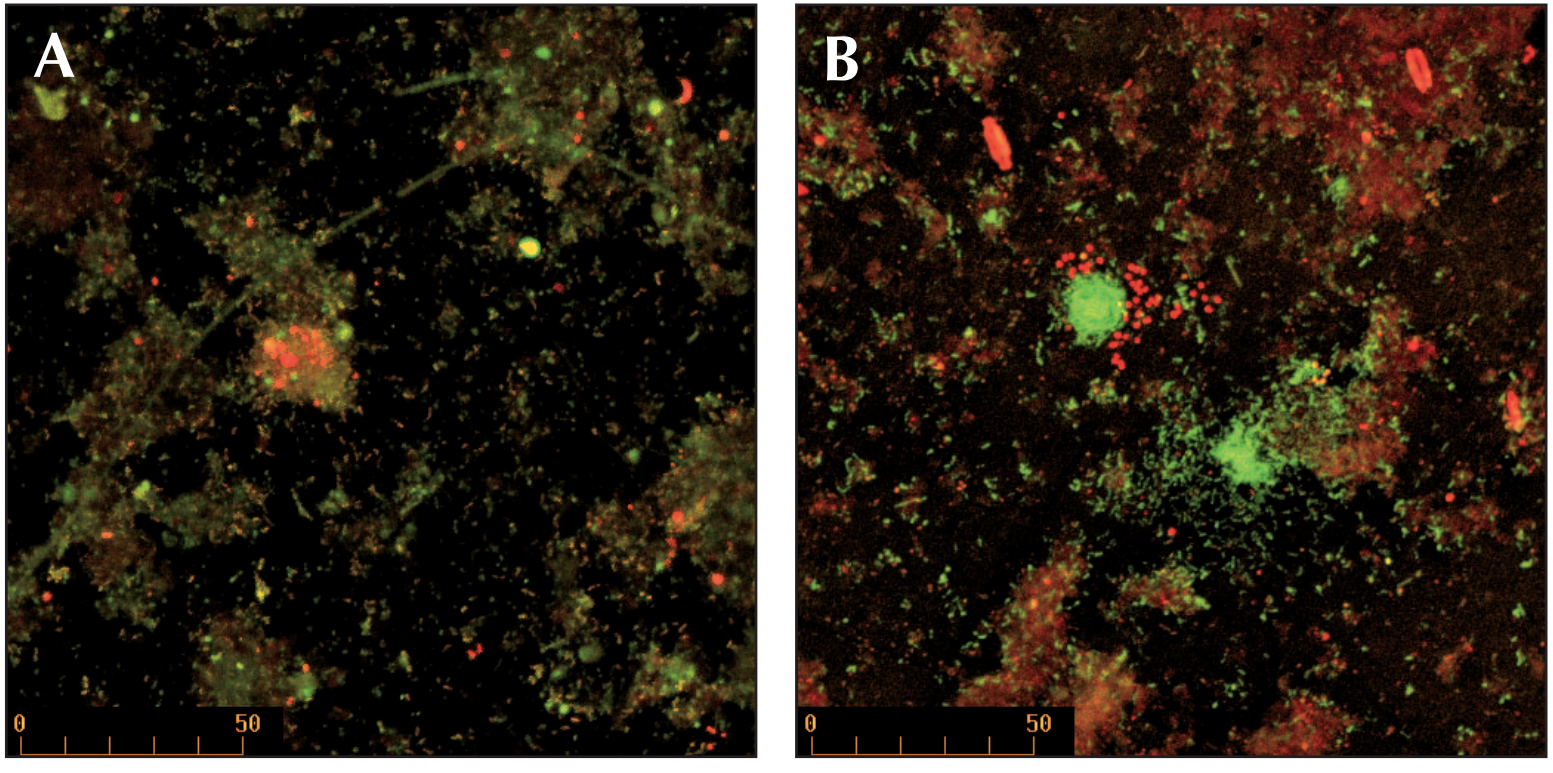


Figure 14. CLSM images of a biofilm on a coupon of stainless steel (UNS S31600), immersed for 30 days in Baltic Sea water in a laboratory mesocosm (≈23° C). The images are pile ups of 20 optical sections, 0.5μm each. A) The image shows a biofilm hybridized with the probe EUB338 (green, targeted for the domain bacteria) and with GAM42a (red, targeted for the gamma group of Proteobacteria). B) The image shows a biofilm hybridised with the probe EUB338 (green, targeted for the domain bacteria) and with ALF1b (red, targeted to alpha group of Proteobacteria). The measure bars are in μm.

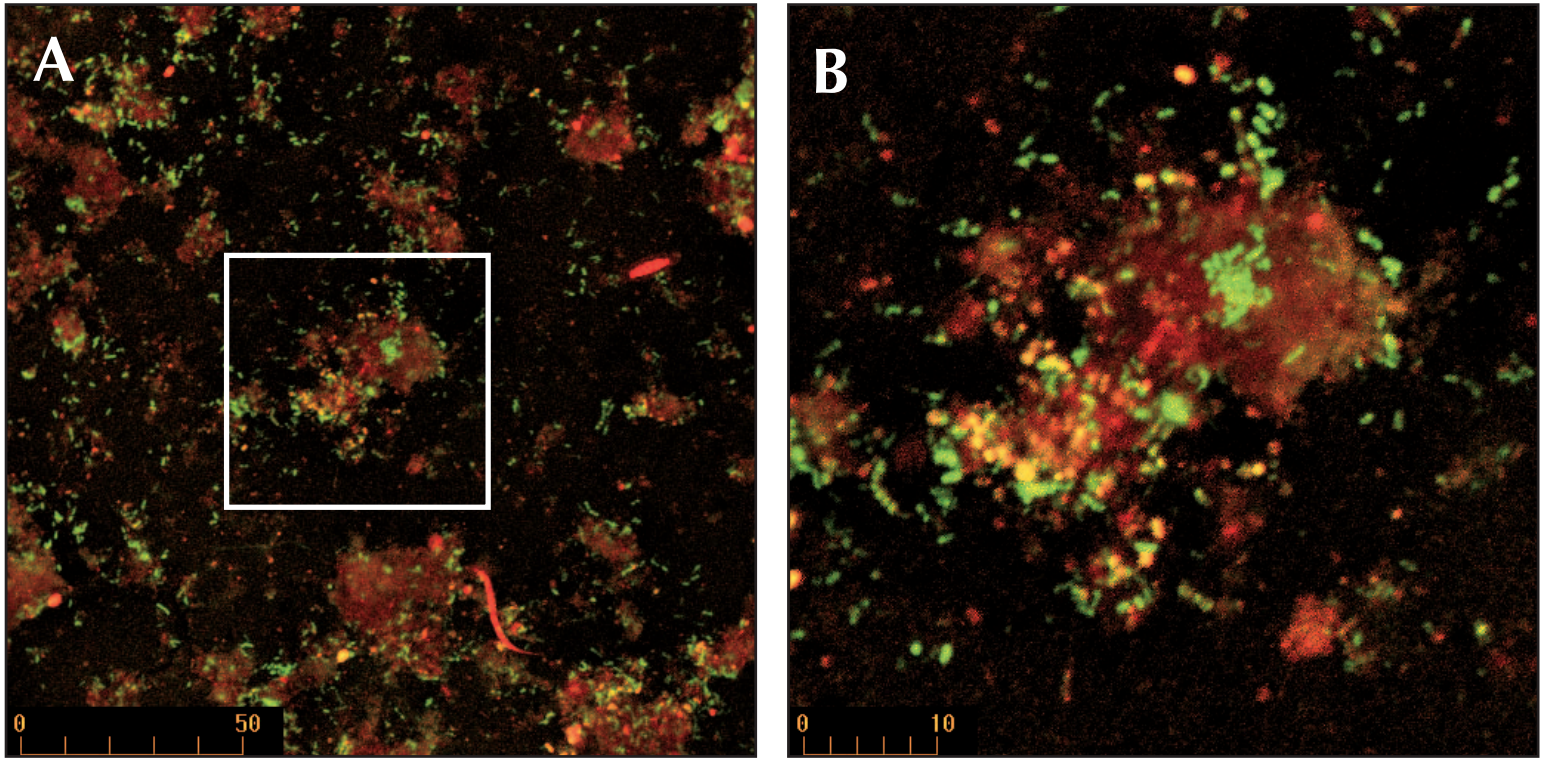


Figure 15. CLSM images of a biofilm on a coupon of stainless steel (UNS S31600), grown for 30 days in Baltic Sea water in a laboratory mesocosm (flow speed ≈30 mm s^{-1}, ≈23° C). The images are pile ups of 20 optical sections, of 0.5 µm each. A) The biofilm hybridized with the probe EUB338 (green, targeted for the domain bacteria) and with probe ALF1b (red, targeted for alpha group of Proteobacteria). B) A zoom up of the central area marked in panel A. Panels A and B show that bacteria with green fluorescent labels (EUB338) were a majority but bacteria with red (ALF 1b) and yellow (double labeled with EUB338 and ALF1b) fluorescence were also seen. The measure bars are in µm.

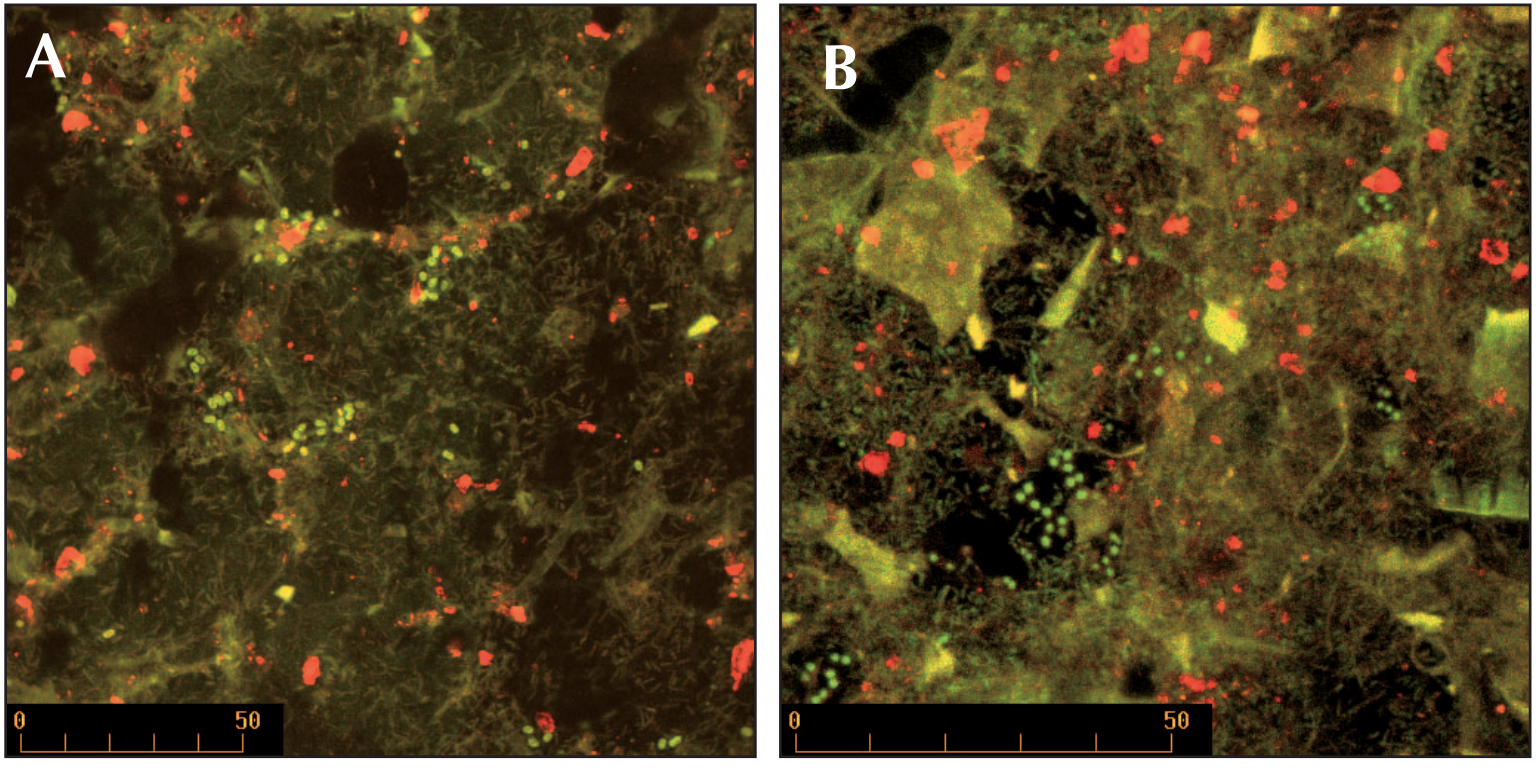


Figure 16. CLSM images of a biofilm on a coupon of stainless steel (UNS S31600) exposed for 50 days in the splash area of the wire section of a printing paper machine. A) A biofilm hybridized with the probe SRB358 (red, targeted to the delta group of Proteobacteria) and with DTM229 (green, targeted to the gram positive genus Desulfotomaculum). B) A biofilm hybridized with the SRB358 (red) and the EUB338 (green, targeted to the domain bacteria) probes. A group of green fluorescing bacteria is visible among the red autofluorescing fines and fibres. Measure bars are in μm.

cell clusters and kill the cells forming the platform of the whole cluster. We used fluorescent stains of different molecular sizes and of different surface properties to assess the permeability properties of the biofilm clusters. As a tool to assess biofilm porosity, we used fluorescently labeled latex beads with a diameter of ≈0.02 μm, i.e. smaller than any of the bacteria in the biofilms. The purpose was to detect whether there were flow channels inside the biofilm and to determine the general porosity of the biofilm clusters.

The biofilm clusters grown during the ennoblement of stainless steel in Baltic Sea water were poorly (≤10 cell layers, Paper II) penetrable to concanavalin A (hydrophilic stain, Table 6) and also to stains (Table 6) with different polarity properties: acridine orange (log K_{ow} 1.24, fig 17), ethidium bromide (log K_{ow} -0.38) and SYTO™ 16 (log K_{ow} 1.48) (Kolari et al 1998). All these substances were excluded from the cores of the biofilm clusters. Autofluorescing diatoms were visible (Fig 17, Fig 6 in Paper I) underneath the biofilm clusters, indicating that the laser beam had penetrated down to the steel surface. The results support the view that biomass with a high diffusion barrier but good penetration of the laser beam (Fig 7 and Fig 6f in Paper II) was present inside the biofilm clusters on the ennobled steel. Such biomass may be involved in the ennoblement of stainless steels.

We found that the fluorescent beads of 0.02 μm diameter with hydrophilic or hydrophobic surfaces only weakly penetrated the biofilm grown on stainless steel *in situ* under conditions of high flow (1.8 m s^{-1}) in paper machine spray water for 7 days (Fig 2 in Paper III). The beads penetrated through the uppermost 2 to 3 cell layers in the biofilms with a total thickness of up to 30 μm. In contrast to the beads, the stains SYTO™16, SYTO™9 (Fig. 18a), ethidium bromide (Fig 18b) and propidium iodide (Fig 18a) penetrated into the core space (through 25 to 30 cell layers) of the same biofilm clusters. Figure 19 shows a single optical section at 3 to 9 μm distance from the steel surface of a biofilm formed in the splash area of the paper machine. It shows that the papermaking fines are a major ingredient in the structure of this biofilm. The bacteria (red, stained with propidium iodide) formed cell clusters amidst the fines of pulping origin. As the biofilm was dry at the time of sampling in the paper machine, this may explain why all the bacteria appear dead (red fluorescence).

Baltic Sea biofilms were grown in the laboratory ecosystem under a flow of 30 mm s^{-1} at 23 ºC. The paper machine biofilms were grown under 60 times higher flow rates and at a temperature of 40 to 50ºC. The exposure periods in the Baltic Sea were long, up to 76 days, but in the paper machine only 7 days. The dyes penetrated into the biofilm core in the paper machine grown biofilms. This may be due to the thinner biofilm. The Baltic Sea grown biofilms were older and thicker (up to 120 μm). The high cluster depth of the Baltic Sea biofilms may have limited the diffusion. Figure 17b shows a pile up of optical z sections of a 120 μm thick Baltic Sea biofilm where acridine orange had penetrated down to a depth of 10 to 25 μm from the surface of the biofilm. The penetration depth of the dyes (ethidium bromide, acridine orange and SYTO™16) was thus similar in the paper machine and in the Baltic Sea water biofilms, indicating a similar penetration resistance. The limited thickness of the paper machine biofilms allowed penetration through the total thickness of the biofilm.

A high flow rate (> 1.5 m s^{-1}) has been considered to protect against fouling and against microbially induced corrosion (MIC) (Geesey 1993). Stoodley et al (1999) found that a flow velocity of 0.7 to 1.3 m s^{-1}

limited the thickness of the biofilm formed on glass in minimal salts medium with glucose as the carbon source to ≈20 μm. Our results showed that a high flow rate (1.8 m s^{-1}) limited the biofilm thickness (max 30 mm, exposure time up to 12 d). According to Vieira and Melo (1999), the thin biofilms formed under high flow rates should be easily penetrated by the biocides used for limiting biofilm growth due to the short diffusion distance.

Conclusions about biofilm formation observed by CLSM

The exclusion of stains and latex beads of 0.02 μm in diameter by the Baltic Sea biofilm indicates that a barrier exists inside the cell clusters grown during the ennoblement of steel. The stains with a small molecule size penetrated down to ≈20 cell layers, but did not reach the steel surface. The high penetration resistance of the biofilm may also be valid for oxygen, leading to the formation of differential aeration cells. Such a situation would permit anaerobic metabolism in the deeper layers of the cluster, closest to the stainless steel surface. The formation of differential aeration cells paves the way for the generation of anaerobic metabolites, such as reduced sulphur-containing anions. Differential aeration may increase the open circuit potential of steels, leading to ennoblement and, subsequently, to corrosion of the steels.
Fluorescent stains penetrated throughout the biofilms grown in a high flow in paper machine water. The mechanisms of steel corrosion in paper machines may be different from those in Baltic Sea water.

4.3 Methods developed in this study

We developed, through trial and error, a novel type of flow cell for use in investigating the biofouling of steels in an industrial environment. The design is shown in Figure 1 of Paper III. The side panels of the flow cells were made of polyacryl, and the top and bottom panels of stainless steel. Sample holders for testing were fitted into the top and bottom panels. The transparent polyacrylic side panels served to allow visual inspection of the flow cell interior without having to remove the samples.

The flow cell was directly connected to the side flow (average 1.8 m s^{-1}) of the water circulation system of a paper machine and operated under machine pressure, with no need for pumps. The flow cell operated continuously for up to 3 weeks without removal for cleaning. The structure of the flow cell allowed separation of the top and bottom panels from the sidewalls for intensive cleaning between experiments. The exchangeable coupons of stainless steel made it possible to compare the biofouling tendency of different types of steel and of different grades of surface finishing. The flow contact surface of the steel coupons had a diameter of 25 mm. Up to 16 coupons could be placed in each flow cell. This made time-lapse studies possible. The flow cells thus proved to be efficient tools for determining the relationship between steel surface smoothness and the tendency to biofoul in a paper machine. The results demonstrated that 500 grit steel was less sensitive to biofouling compared to 100 grit steel (Fig 9 and 10 in Paper III).

Scanning fluorometers are available on the market for monitoring microtiter plates. We devised a protocol for using this instrument for direct scans of the biofilms on stainless steel. To accomplish this, the biofilms on the steel coupons were stained with a fluorescent dye. The coupons were then placed in the wells of microtiterplates, one coupon in each of the 6 wells. The scanning area of the fluorometer was programmed

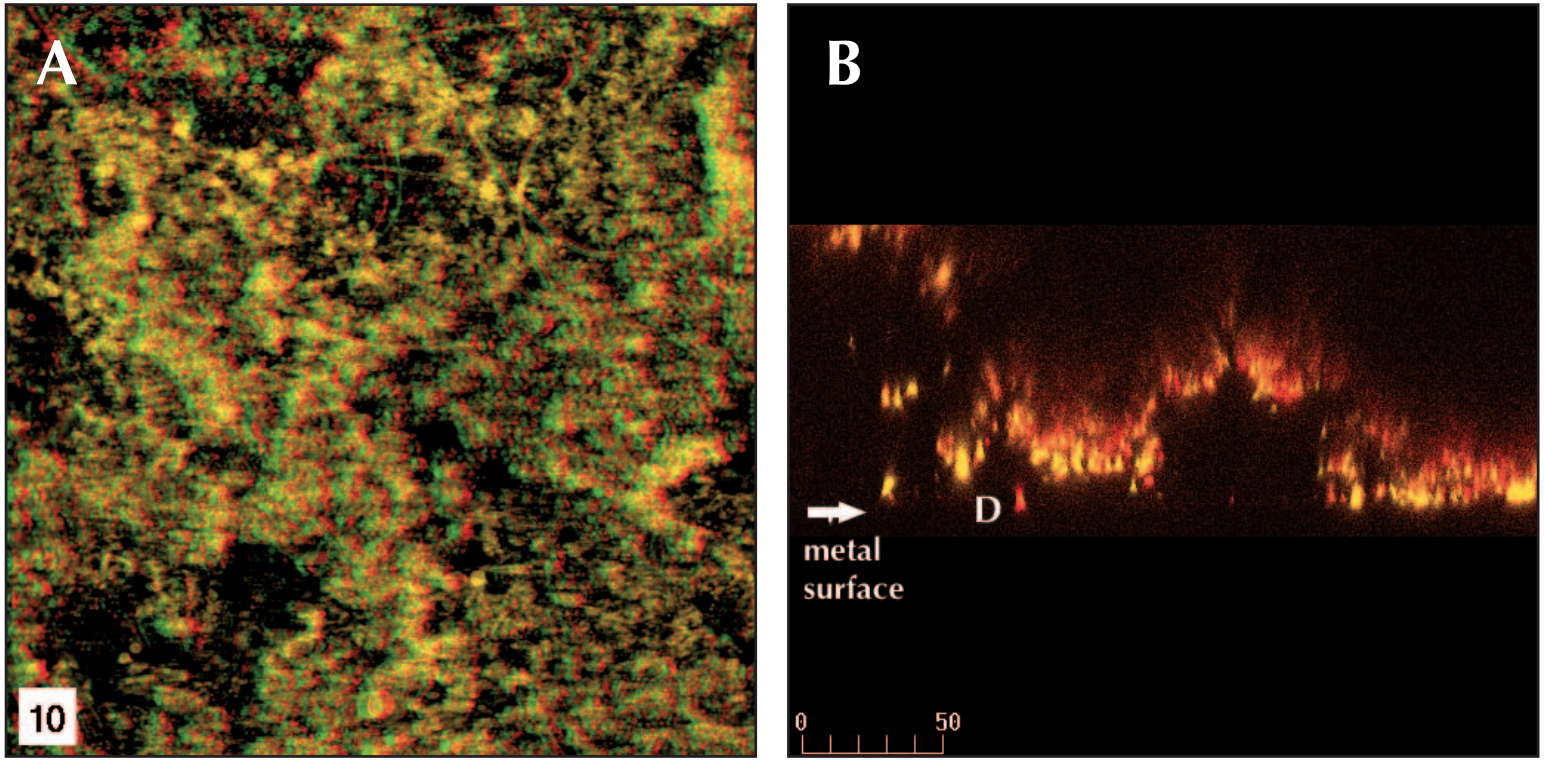


Figure 17. Confocal laser scanning microscopy (CLSM) images of an acridine orange stained biofilm on a stainless steel (UNS S31600) coupon grown for 76 days in Baltic Sea water in the laboratory mesocosm (very low flow speed, ≈23ºC). A) 3D stereo image of a pile up of 250 individual optical sections (0.5 µm thick each) of a biofilm. B) A z-section of same pileup. The image shows that acridine orange only had penetrated into the depth of 10 to 25 µm corresponding to approximately 10 to 20 layers of cells. Measure bars are in µm. (D= a diatom)

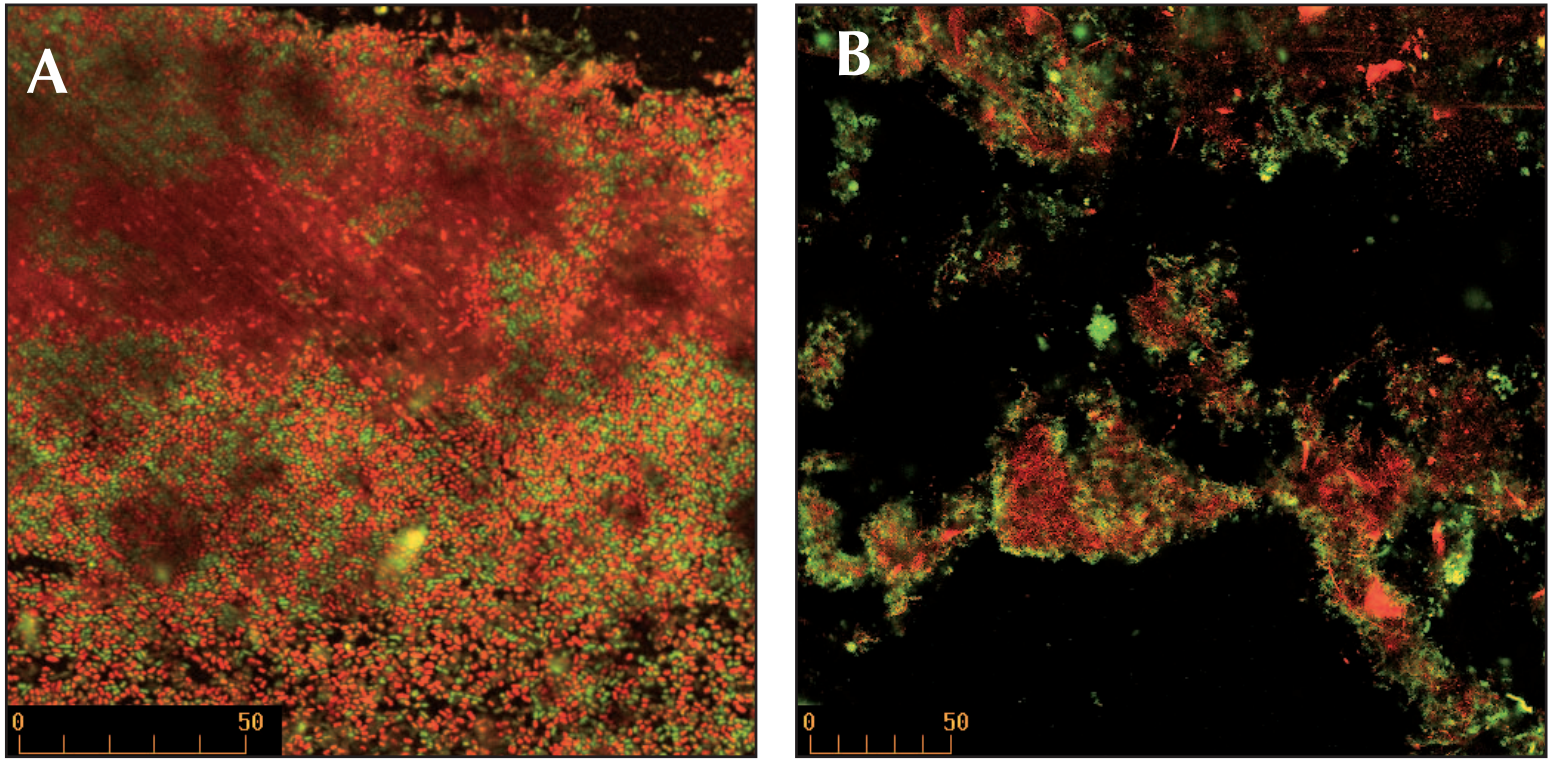
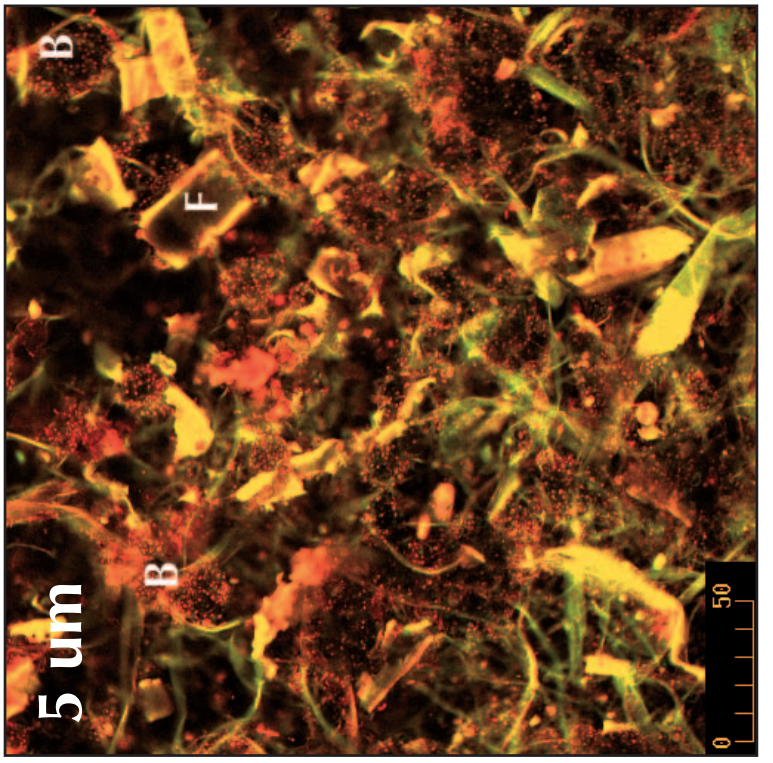


Figure 18. CLSM images of a biofilm on a coupon of stainless steel (UNS S31600) exposed for 5 days in the spray water circuit of a printing paper machine (≈45ºC). A) A single optical section (1 μm) of the biofilm, viability stained with syto 9 (live/green) and propidium iodide (dead/red). The image is taken from the layer immediate to the steel surface. A majority of the bacteria in the periphery of the biofilm cluster are red staining dead bacteria. B) A single optical section (1 μm) of a biofilm stained with ethidium bromide (red) and carboxylate-modified fluorescent beads (diameter 0.02 μm, green). The image was taken at the distance of 6 μm to steel surface. The figure shows that these fluorescent beads penetrated to the peripheral parts of biofilm cluster only. Measure bars are in μm.

to match the area of the surface of the stainless steel coupons. The emitted fluorescence was recorded from 137 points on each coupon (diameter 25 mm), and expressed as relative fluorescence units (rfu). The background autofluorescence of each individual coupon was premeasured before placing the coupons in the flow cells. This ensured that minor differences in the finishing of the individual steel coupons would not interfere with results of the biofouling measurements.

This novel method permitted the rapid acquisition of quantitative and qualitative information about a living biofilm while still attached to its original substratum, as well as the use of different stains. The fluorescent methods gave relevant information especially at the time when the coupon surfaces were not yet fully covered by the biofilm. Scanning fluorometry is less suitable for the analysis of thick biofilms.

The biofilms formed on a steel surface under paper machine conditions (high flow, hostile aquatic environment) were thin (≤ 30 μm, in 7 days). Their ATP content was too low to be accurately measured by the protocols described in the literature. We developed a novel extraction protocol for measuring ATP in these biofilms (Paper III). It involved inserting the steel coupons with the biofilm face downwards into 5 ml of boiling Tris-EDTA with glass beads. During the 5 min of boiling, the glass beads mechanically disintegrated the cell clusters tightly bound to the steel, releasing the ATP into the buffer where it could be analysed by the luminometric method (Fig 9 and 11 in Paper III). The results showed that it was possible (detection limit 10^2 cells cm^{-2}) to extract biofilm ATP from biofouled stainless steel surfaces, and to use the result as an indicator of biofouling on paper machine steels (Paper III).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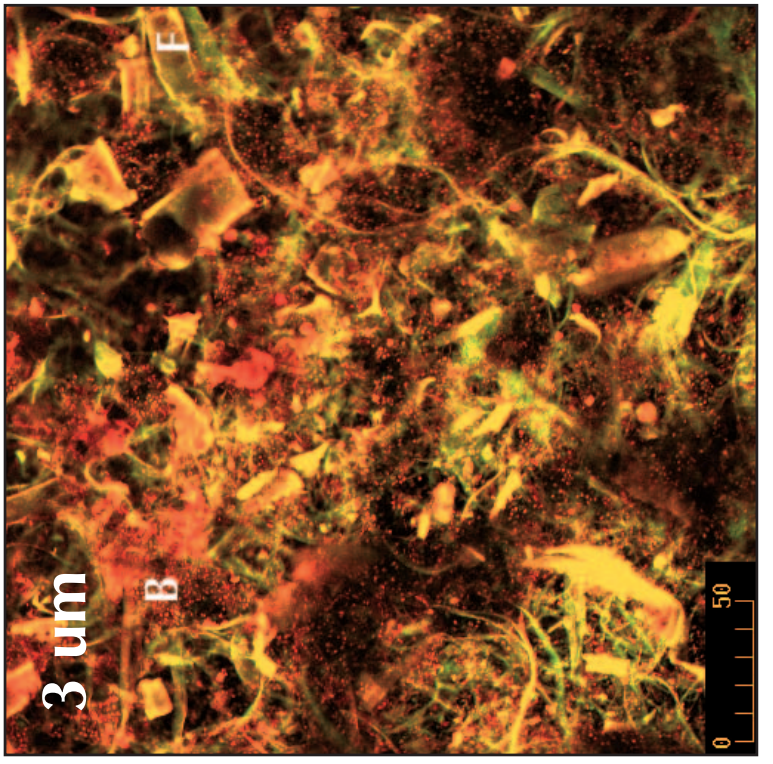
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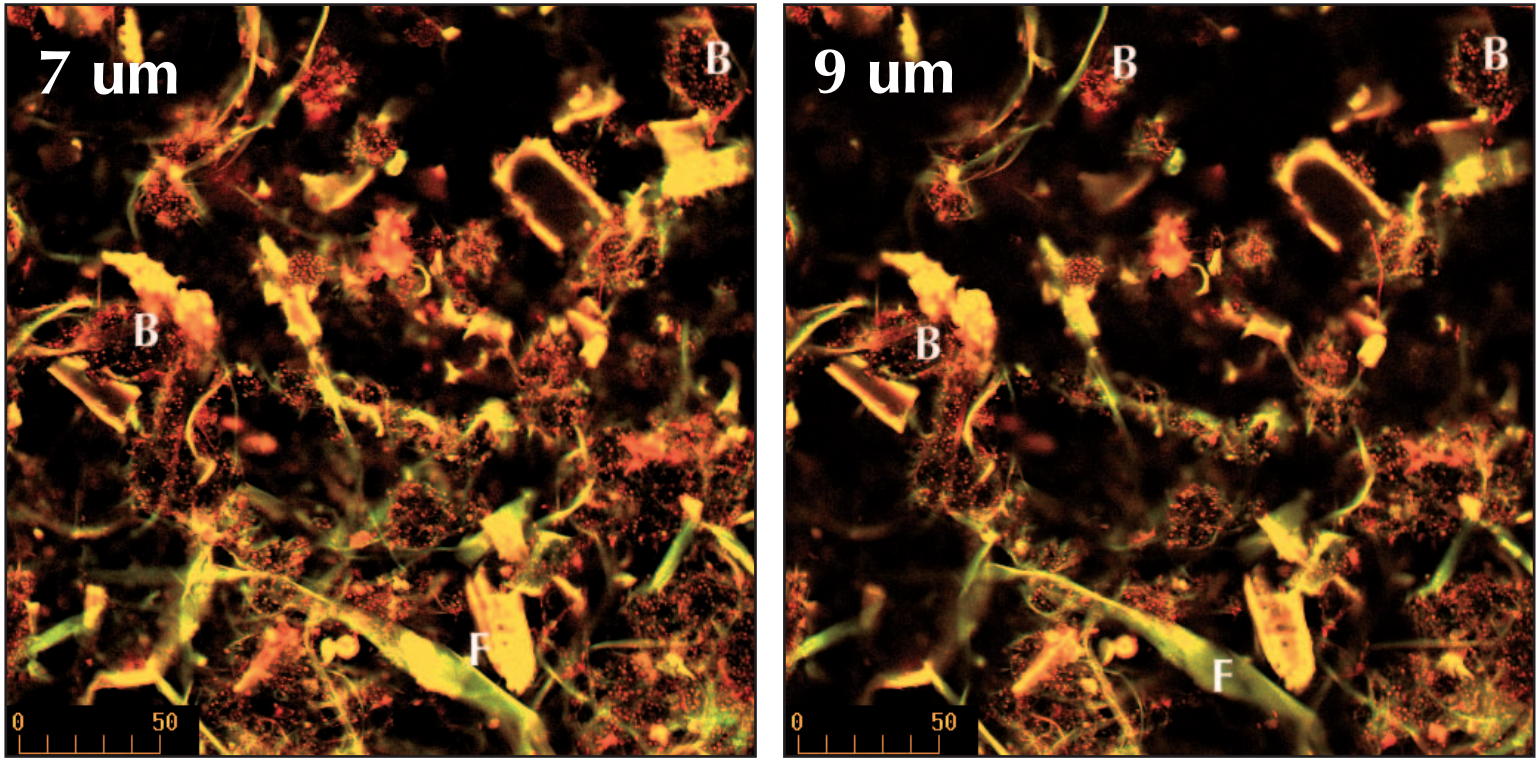

Figure 19. CLSM images of a biofilm on a coupon of stainless steel (UNS S31600) esposed for 50 days in the splash area of the wire section of a printing paper machine. Distances of the respective optical sections from the steel surface are indicated. The biofilm was viability stained with syto 9 (live/green) and propidium iodide (dead/red). Red fluorescencing bacterial (B) aggregates are visible among the paper machine fines and fibres (F). Most particles visible in the images show autofluorescence. Measure bars are in μm.

5 Summary and conclusions

I

Biofilm formation in Baltic Sea water during the ennoblement of stainless steel started with the attachment of single bacteria. SEM inspection showed *Seliberia, Hyphomicrobium* and *Caulobacter*-like bacteria attached to the steel surface underneath a thin layer of presumably organic substances. Ennoblement of the stainless steels occurred well before the biofilm clusters had fully covered the steel surface. This indicated that the ennoblement of steel may be associated with the coexistence of patches with varying access to oxygen. The patchy, electrically insulating biofilm may form the basis for the onset of corrosion. We were able to *in situ* hybridise biofilm-forming bacteria from the steel surface. The majority of the biofilm-forming bacteria in the Baltic Sea belong to the domain bacteria and/or alfa proteobacteria.

II

The ennoblement of stainless steel reproduced in a laboratory mesocosm was similar to that occurring in the Baltic Sea. The oligotrophic brackish water allowed ennoblement to occur in the mesocosm at a flow rate of between 10 and 35 mm s^{-1}. At low (0ºC) water temperature, ennoblement in the Baltic Sea occurred within 50 days, and at 23ºC within 20 to 30 days. A water temperature of 32ºC reversibly inhibited the onset of ennoblement in the laboratory mesocosms: ennoblement resumed when the temperature was decreased to 26ºC. A two fold increase in the content of nutrients in the Baltic Sea water used as the feed inhibited the onset of ennoblement. An increase in the nutrient content also changed the structure of the biofilm.

III

We showed, using a paper machine splash area simulator, that thiosulphate and oxalate were generated *de novo* from paper pulp and simulated white water. The materials placed in the simulator before the experiment, or the simulated white water used as the feed, contained no thiosulphate or oxalate. The simulator contained sheets of pulp that were preinoculated with strains of *Bacillus, Desulfovibrio* and *Desulfotomaculum*, as well as an unidentified SRB culture from a paper machine environment. Corrosion pits were formed on the stainless steel facing the pulp sheets. The pits occurred in areas where large amounts of thiosulphate and oxalate had accumulated in the pulp sheets.

IV

We constructed flow cells and used them successfully for monitoring biofilm formation *in situ* in a paper mill. The flow cells were attached to the spray water circuit of the machine. The biofilm clusters formed under these high flow conditions consisted of bacteria with a uniform shape and size within each cluster, but with varying shapes and/or sizes between the clusters. Cocci, and short and long rod-shaped bacteria were present in the biofilms, but no fungi or other eukaryotes. Latex beads with a diameter of 0.02 µm penetrated through only 2 to 3 layers of cells in the biofilm clusters. In contrast to the beads, SYTO™ 16, SYTO™9, ethidium bromide and propidium iodide stains penetrated into the core of the 30 µm-thick biofilm clusters.

6 Tiivistelmä

Itämeren vedessä ja paperikoneen prosessivedessä mikrobien tarttuminen ruostumattomaan teräkseen käynnistää biofilmin muodostumisen. Se voi pahimmillaan johtaa putkistojen tukkeutumiseen, prosessin teho laskuun, korroosion syntyyn jne.

Itämeren vedessä ensimmäisiä teräkseen tarttuvia mikrobisukuja olivat *Seliberia, Hyphomicrobium* ja *Caulobacter*. Näiden lisäksi metallin pinnalle muodostui ohut orgaanisesta aineesta muodostunut kalvo joka peitti mikrobit. Luonnonvedelle altistetun ruostumattoman teräksen lepopotentiaali kasvoi ajan myötä. Tätä tapahtumaa kutsutaan jalontumiseksi. Jalontuminen tapahtui jo silloin kun teräksen pinta oli vasta osittain biofilmin peittämä. Osittainen peittävyys saattaa aiheuttaa eroja hapen pääsylle metallin pintaan ja näin luoda edellytykset korrosion synnylle. *In situ* hybridisaatio menetelmällä havaitsimme että suuri osa biofilmimikrobeista oli bakteereja, erityisesti alfaproteobakteereja.

Teräs jalontui Itämeren vedessä laboratoriossa samalla tavoin kuin meressä. Jalontuminen edellytti riittävän suurta virtausnopeutta (10-35 mm s^{-1}). Jalontumiseen tarvittu aika oli pidempi (50 d) kylmässä (0ºC) kuin lämpimässä merivedessä (20-30 d, 23ºC). Meriveden lämpötilan nosto 32ºC:n esti jalontumisen alkamisen mutta lämpötilan laskettua jalontuminen käynnistyi. Myös ravinne pitoisuuksien kaksinkertaistaminen merivedessä esti jalontumisen ja muutti biofilmin rakennetta.

Paperikoneen märän pään roiskevyöhykettä simuloivassa laitteessa muodostui tiosulfaattia ja oksalaattia synteettisestä paperikonevedestä ja selluloosasta bakteerien (*Bacillus*, paperikone SRBt, *Desulfovibrio* ja *Desulfotomaculum*) läsnäollessa. Tiosulfaattia tai oksalaattia ei ollut, tai oli vain pieniä määriä vedessä tai sellussa. Märkien selluloosaarkkien alla olleeseen ruostumattomaan teräkseen tuli syöpymiä alueille joiden päällä olleesta sellusta löytyi oksalaatia ja tiosulfaattia korkeina pitoisuuksina.

Biofilmin muodostumisen tutkimiseksi paperikoneessa valmistettiin virtauskennot. Kennot kytkettiin viiran suihkuveden vesikiertoon. Voimakas virta (1.8 m s^{-1}) rajoitti biofilmi klustereiden korkeutta. Muodostuneet klusterit koostuivat useimmiten vain yhdenlaisista bakteereista. Bakteerien koko ja muoto vaihtelivat klusterista toiseen. Muodostuneiden biofilmiklustereiden tiiviyttä tutkittiin mittaamalla väriaineiden tunkeutumissyvyyttä. Lateksikuulat (halkaisija 0.02 μm) tunkeutuivat vain 2-3 solukerroksen syvyyteen. Fluoresoivat väriaineet SYTO™16, SYTO™9, etidiumbromidi ja propidiumjodidi tunkeutuivat 30 μm paksun biofilmin läpi.

Väitöskirjatyö osoitti, että biofilmejä voi menestyksellä tutkia suoraan teräspinnoilta, joille mikrobit ovat kasvaneet alkuperäisissa olosuhteissa, kuten tehtaassa tai meressä. Aiempi tietämys biofilmeistä perustui yleensä laboratoriokasvatuksiin lasi- tai muovipinnoilla. Tässä väitöskirjassa kehitetyt menetelmät ja tuotettu tieto biofilmien mikrobirakenteesta ja erilaisten aineiden tunkeutuvuudesta niihin. Tulokset antavat luotettavan pohjan prosessiteollisuuden tuotantolaitteiden pesuohjelmien suunnitteluun, etenkin cleaning-in-place tilanteissa, ja myös mikrobikasvua estävien kemiallisten aineiden ja fysikaalisten menetelmien kehittämiseen.

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